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AORTA LIPID AND PROTEIN CHANGES IN RATS DURING AND AFTER FEEDING A LOW-PROTEIN DIET. ROLE OF THE THYROID GLAND

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ABSTRACT

Young male rats were fed standard (normal protein content) or low-protein diets for four weeks. Protein-deficient rats were refed the standard diet for another two or four weeks. Thyroidectomy was performed in the beginning of the experiment or in the beginning of the protein-recovery period. Total cholesterol, triglycerides, phospholipids, total protein and hydroxyproline levels decreased in aorta of rats receiving the low-protein diet. Thyroidectomy in these animals promoted increase of lipid content while decreased protein levels in aorta. The alterations promoted by the low-protein diet disappeared when the protein supply was restituted by diet; but, in the thyroidectomized animals the same occurred only when triiodothyronine was together supplied in physiological doses. It was concluded that physiologically active levels of T_3 could contribute for the lower aorta lipid levels in low-protein fed rats and that the recovery of a normal aorta metabolism after a protein undernutrition period is not possible without a normal thyroid function.

INTRODUCTION

The role of nutritional factors such as the type of dietary fat, the type of carbohydrate and even of mineral elements in the development of atherosclerosis has been widely emphasized (1). Dietary protein has been implicated as a contributory factor to plasma and arterial wall lipid changes (2), even when normocaloric conditions were kept (3,4). The effect of protein refeeding after a consumption period of low-protein diet, on plasma and aortic lipid levels was also described (5). However, the role of hormonal factors in protein related changes in aortic metabolism is unknown. There are evidences suggesting that the elevated plasma triiodothyronine concentration of rats fed on protein deficient diets is physiologically active (6). But little is known about the importance of thyroid hormones during and after a protein undernutrition period on aortic metabolism. To obtain more information on this subject, studies of lipid and protein constituents in the plasma and aortas of normal and thyroidectomized rats were carried out during and after protein restriction by diet.

MATERIALS AND METHODS

Diets

Two isocaloric (471 kcal/100 g) diets were used: a standard-protein diet (27.6% of protein) and a low-protein diet (2.6% of protein). Both diet compositions were given elsewhere (7).

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Briefly, the composition of the standard-protein diet was: corn starch (29%), sucrose (10%), wheat flour (10%), soybean oil (12%), powdered milk (10%), casein (25%), brewer's yeast (3%), and saline mixture of Osborne-Mendel (1%). Liposoluble vitamins were added: A (2,000 IU), D (200 IU), E (11 mg) and K (5 mg/100 g of food). Further additions, per 100 g of food: thiamin hydrochloride (2.19 mg), riboflavin (2.19 mg), niacin (9.9 mg), calcium pantothenate (6.6 mg), pyridoxine (2.19 mg), biotin (0.044 mg), folic acid (0.198 mg), cyanocobalamine (0.003 mg), inositol (10.99 mg), choline (165 mg) and p-aminobenzoic acid (11 mg).

The low-protein diet was casein-free and its protein content was derived only from the powdered milk. Equivalent calories were replaced by soybean oil and cellulose was added to increase the bulk. This low-protein diet was considered as good experimental model to study the effects of varying levels of protein intake without changing total plasma cholesterol, triglycerides and phospholipids (7).

Design and Analysis

Twenty-one-day-old Sprague-Dawley rats, weighing 40-45 g were used. Immediately after weaning, the rats were separated into the following groups: (a) the standard-protein group, which received the standard protein diet for 4, 6 or 8 weeks (groups S_4 , S_6 and S_8 , controls), (b) the low-protein group, which received the low-protein diet during four weeks (group L), and (c) the thyroidectomized-low-protein group, which rats thyroidectomized at weaning (three weeks old) received the low protein diet during four weeks (group TL). At the end of the fourth week of treatment, some rats of groups L and TL were refed the standard-protein diet for two or four weeks (groups $L \rightarrow S_2$, $L \rightarrow S_4$, $TL \rightarrow S_2$ and $TL \rightarrow S_4$). Some animals of L group were also thyroidectomized in the beginning of refeeding the standard-protein diet (groups $L \rightarrow T_2$ and $L \rightarrow T_4$). Finally, it was administered, during the refeeding of the standard-protein diet, to some animals of TL group, physiological doses of triiodothyronine (T_3), 0.5 ug/100 g body weight SC daily (groups $TL \rightarrow [T_3]_2$ and $TL \rightarrow [T_3]_4$), during two or four weeks. This dose has maintained the normal plasma levels of T_3 and cholesterol.

Rats were allowed free access to food and water, weighed once a week, and kept on a 10-hour light, 14-hour dark cycle at 23°C room temperature.

At the end of each experimental period, rats were fasted for 15 h and then bled through aorta under light ether anesthesia. Plasma was separated by centrifugation and analyzed for total cholesterol (8) and total protein (9). Aortas were washed with cold 0.157 M KCl, and cleaned mechanically. After extraction for Collagen (10) and lipids (11), total cholesterol (8,12), phospholipids (13), triglycerides (14), hydroxyproline (15) and total protein (9) were determined in these extraction solutions.

Statistical analysis was carried out using Student's t test. Since the animals fed the standard-protein diet during 4-8 weeks showed no significant differences regarding the biochemical parameters studied, all these animals were pooled into one single group (S).

RESULTS

Table 1 presents the body weight gain (weight at the end of the experiment minus the weight at the beginning) of the rats of each group. Weight gain of animals fed the standard-protein diet increased constantly and directly related to the length of the experimental period (S_4 to S_8). On the other hand, animals fed the low-protein diet for four weeks (groups L and TL)

TABLE I

Effects of standard-and low-protein diets administered for varying periods of time, and influence of thyroidectomy (see text) on Δ body weight (final body weight minus initial body weight).
Values are mean \pm SEM.

GROUP	Δ WEIGHT (g)	GROUP	Δ WEIGHT (g)	GROUP	Δ WEIGHT (g)
S_4	$105.20 \pm 5.20(6)^{ax*}$	S_6	$140.33 \pm 5.03(6)^{ay}$	S_8	$216.00 \pm 4.37(6)^{az}$
L	$10.22 \pm 0.90(10)^{bx}$	L \rightarrow S_2	$70.21 \pm 4.80(10)^{by}$	L \rightarrow S_4	$125.42 \pm 5.59(10)^{bz}$
		L \rightarrow T_2	$49.30 \pm 2.42(10)^{cy}$	L \rightarrow T_4	$90.17 \pm 6.30(10)^{cz}$
TL	$10.86 \pm 6.02(10)^{cx}$	TL \rightarrow S_2	$67.30 \pm 3.90(10)^{by}$	TL \rightarrow S_4	$100.32 \pm 5.80(10)^{cz}$
		TL \rightarrow $(T_3)_2$	$75.40 \pm 4.20(10)^{by}$	TL \rightarrow $(T_3)_4$	$133.75 \pm 6.30(10)^{dz}$

*letters a, b, c and d refer to values in each column; letters x, y, and z refer to values in each line.
Different letters mean $p < 0.05$; identical letters mean not statistically different.
Numbers in parentheses = number of rats

had a minimal increase in body weight. This situation was reverted by refeeding the standard-protein diet, but without reaching the weight of standard-protein group animals, even at the end of the fourth week. Moreover, the thyroidectomized groups presented the lowest weight gain during refeeding, while T_3 administration prevented the results caused by the thyroid gland absence.

In Table 2 it can be seen that the low-protein diet (group L) caused a decrease in all measured parameters in aorta, while decreased the plasma protein content and maintained the plasma total cholesterol level. When the low-protein diet was supplied to the thyroidectomized rats (group TL), the lipid content in aorta increased, while, in comparison to L group, hydroxyproline levels decreased and aorta total protein concentration was maintained at the same level. In plasma, there was an increase in total cholesterol in the TL group, while plasma protein concentration also remained at the same levels that those observed in the L group.

In Table 3 it can be seen the effect of refeeding the standard-protein diet after the low-protein diet period and the influence of thyroidectomy during this recovery period. When rats were refed for two weeks ($L \rightarrow S_2$) the standard protein diet after the low-protein diet period, there was an increase in the aorta lipid content and in plasma cholesterol levels above control levels. Aorta hydroxyproline and total protein and proteinemia also increased, but remained below control levels. At the fourth week of refeeding ($L \rightarrow S_4$) all the parameters returned to control values, with the exception of aortic hydroxyproline, that remained low. Rats thyroidectomized in the beginning of refeeding presented, at the second week ($L \rightarrow T_2$), decreased levels of aorta hydroxyproline and an increase in other measured parameters, the aorta lipids and cholesterolemia being higher than control values. Differently from what occurred in non-thyroidectomized rats, these lipid levels continued to increase until the fourth week of refeeding ($L \rightarrow T_4$), while aorta hydroxyproline and total protein and proteinemia remained lower than control values.

In Table 4 it can be seen the effect of refeeding standard-protein diet after a low-protein diet period in thyroidectomized rats, and the influence of T_3 administration. The refeeding of the standard-protein diet for two weeks ($TL \rightarrow S_2$) did not change the measured parameters presented in TL group, with the the exception of aorta phospholipid an total protein levels that have increased. But, at the fourth week of refeeding ($TL \rightarrow S_4$), all these parameters increased, resulting in the great content of aorta lipid and plasma cholesterol levels; aorta hydroxyproline and aorta an plasma total protein levels, although increased in comparison to TL group, remained at levels below control. These facts did not occur when T_3 was administered together to the refeeding diet. The lipid content in aorta and the cholesterolemia decreased, and aorta and plasma total protein increased, reaching control levels at the fourth week of refeeding ($TL \rightarrow [T_3]_4$). Hydroxyproline levels, although have increased, did not reach control levels at that time.

DISCUSSION

Rats fed the low-protein diet (L) had a very poor appearance. Besides, they had livers with macroscopic signs of steatosis. These facts, together with the low proteinemia and the low growth rate of these animals, stated that a severe degree of undernutrition was obtained.

Although the low-protein diet (L) promoted, as expected, a decrease in plasma protein concentration, it appeared to have no significant effect on serum cholesterol, as it has been previously shown (5). These rats showed a

TABLE II

Aorta concentration of total cholesterol, phospholipids, triglycerides, hydroxyproline and total protein and plasma levels of total cholesterol and total protein of rats fed standard-protein (S) or low-protein diets (L) and thyroidectomized rats fed low-protein diet (TL). Each value represents mean \pm SEM.

GROUP	AORTA (mg/g dry weight)				PLASMA	
	TOTAL CHOLESTEROL	PHOSPHOLIPIDS	TRIGLYCERIDES	HYDROXYPROLINE	TOTAL CHOLESTEROL (mg/dl)	TOTAL PROTEIN (g/dl)
S(18)	2.60 \pm 0.24 ^{a*}	2.27 \pm 0.20 ^a	2.23 \pm 0.13 ^a	4.03 \pm 0.20 ^a	82.26 \pm 7.45 ^a	7.42 \pm 0.20 ^a
L(10)	2.10 \pm 0.15 ^b	1.80 \pm 0.15 ^b	1.84 \pm 0.12 ^b	3.01 \pm 0.16 ^b	85.90 \pm 7.30 ^a	6.18 \pm 0.12 ^b
TL(10)	3.94 \pm 0.16 ^c	3.45 \pm 0.19 ^c	3.26 \pm 0.40 ^c	2.50 \pm 0.10 ^c	157.59 \pm 9.57 ^b	6.26 \pm 0.18 ^b

number in parentheses = number of rats

*different letters mean $p < 0.05$; identical letters mean not statistically different.

TABLE III

Aorta total cholesterol, phospholipids, triglycerides, hydroxyproline and total protein and plasma total cholesterol and total protein content of rats refed standard-protein diet after a protein restriction period. Influence of thyroidectomy. Values are mean \pm SEM.

GROUP	AORTA (mg/g dry weight)					PLASMA	
	TOTAL CHOLESTEROL	PHOSPHOLIPIDS	TRIGLYCERIDES	HYDROXYPROLINE	TOTAL PROTEIN	TOTAL CHOLESTEROL (mg/dl)	TOTAL PROTEIN (g/dl)
S(8)	2.60 \pm 0.24 ^a	2.27 \pm 0.20 ^a	2.23 \pm 0.13 ^a	4.03 \pm 0.20 ^a	240.18 \pm 8.15 ^a	82.26 \pm 7.45 ^a	7.42 \pm 0.20 ^a
L(10)	2.10 \pm 0.15 ^b	1.80 \pm 0.15 ^b	1.84 \pm 0.12 ^b	3.01 \pm 0.16 ^b	162.40 \pm 9.17 ^b	85.90 \pm 7.30 ^a	6.18 \pm 0.12 ^b
L \rightarrow S ₂ (10)	4.98 \pm 0.29 ^c	3.98 \pm 0.35 ^c	4.02 \pm 0.48 ^c	3.52 \pm 0.11 ^c	196.29 \pm 7.23 ^c	105.33 \pm 6.86 ^b	6.82 \pm 0.12 ^c
L \rightarrow S ₄ (10)	2.81 \pm 0.16 ^a	2.30 \pm 0.15 ^a	2.32 \pm 0.18 ^a	3.68 \pm 0.16 ^c	230.02 \pm 8.30 ^a	86.31 \pm 7.85 ^a	7.55 \pm 0.20 ^a
L \rightarrow T ₂ (10)	3.64 \pm 0.22 ^d	3.58 \pm 0.20 ^c	2.98 \pm 0.15 ^d	2.30 \pm 0.12 ^d	190.25 \pm 8.40 ^c	150.63 \pm 10.22 ^c	6.25 \pm 0.15 ^b
L \rightarrow T ₄ (10)	4.67 \pm 0.35 ^c	5.90 \pm 0.49 ^d	3.28 \pm 0.31 ^e	2.42 \pm 0.12 ^d	200.02 \pm 8.30 ^c	199.96 \pm 12.42 ^d	6.37 \pm 0.18 ^b

Number in parentheses = number of rats. Different letters mean $p < 0.05$; identical letters mean not statistically different.

TABLE IV

Aorta total cholesterol, phospholipids, triglycerides, hydroxyproline and total protein and plasma total cholesterol and total protein in thyroidectomized rats refed standard-protein diet after a low-protein diet period. Effect of T₃ administration during the refeeding period. Values are mean \pm SEM.

GROUP	AORTA (mg/g dry weight)				PLASMA		
	TOTAL CHOLESTEROL	PHOSPHOLIPIDS	TRIGLYCERIDES	HYDROXYPROLINE	TOTAL PROTEIN	TOTAL CHOLESTEROL (mg/dl)	TOTAL PROTEIN (g/dl)
S(18)	2.60 \pm 0.24 ^a	2.27 \pm 0.20 ^a	2.23 \pm 0.13 ^a	4.03 \pm 0.20 ^a	240.18 \pm 8.15 ^a	82.26 \pm 7.45 ^a	7.42 \pm 0.20 ^a
TL(10)	3.94 \pm 0.16 ^b	3.45 \pm 0.19 ^b	3.26 \pm 0.40 ^b	2.50 \pm 0.10 ^b	175.30 \pm 10.11 ^b	157.59 \pm 9.57 ^b	6.26 \pm 0.18 ^b
TL \rightarrow S ₂ (10)	3.99 \pm 0.15 ^b	4.05 \pm 0.21 ^c	3.69 \pm 0.31 ^b	2.29 \pm 0.15 ^b	198.02 \pm 7.45 ^c	156.80 \pm 11.10 ^b	6.32 \pm 0.10 ^b
TL \rightarrow S ₄ (10)	4.59 \pm 0.29 ^c	4.02 \pm 0.23 ^c	3.82 \pm 0.30 ^b	2.50 \pm 0.18 ^b	200.04 \pm 8.45 ^c	160.43 \pm 8.32 ^b	6.62 \pm 0.15 ^c
TL \rightarrow [T ₃ J ₂ (10)	3.99 \pm 0.21 ^b	2.50 \pm 0.31 ^a	2.80 \pm 0.21 ^c	3.40 \pm 0.15 ^c	210.21 \pm 10.20 ^c	130.25 \pm 8.26 ^c	6.98 \pm 0.12 ^d
TL \rightarrow [T ₃ J ₄ (10)	2.82 \pm 0.12 ^a	2.30 \pm 0.21 ^a	2.10 \pm 0.21 ^a	3.88 \pm 0.10 ^d	245.15 \pm 13.20 ^a	92.42 \pm 8.45 ^a	7.26 \pm 0.13 ^a

Number in parentheses = number of rats. Different letters mean $p < 0.05$; identical letters mean not statistically different.

diminished concentration of total cholesterol, phospholipids, triglycerides, hydroxyproline and total protein in the arterial wall, that has also been demonstrated in our previous reports (3,5).

It is well known that thyroidectomy enhances plasma lipid concentration (17); the resulted hypercholesterolemia seems to be due to a decreased excretion and catabolism of cholesterol (16). These facts seem to be independent of the protein level intake (17). It was also shown that the aorta concentration of total cholesterol, phospholipids, and triglycerides increased, while aortic content of total protein, hydroxyproline and plasma protein levels decreased in thyroidectomized rats (17). The same occurred in this work when thyroidectomized rats were fed the low-protein diet. The decrease observed in plasma and aorta total protein levels seems to be independent of the presence of thyroid gland, while thyroidectomy in the rats fed the low-protein diet (LC group) produced a more marked decrease in aorta hydroxyproline than that observed in L group.

It was shown that animals fed on low-protein diet had elevated plasma concentrations of both total and free triiodothyronine (6,17) physiologically significant (6). This could be consistent with the decreased lipid levels observed in aorta of animals of L group. It was also shown that the hypoalbuminemia seen in rats given less than optimum levels of protein developed as a consequence of the animal's metabolic response to its relative excess energy consumption rather than to protein deficiency per se (18); these animals also consume excess amounts of energy and dispose of most of it by thermogenic mechanisms. This could then contribute for the low levels of aortic hydroxyproline and protein observed in animals of L group. These hypothesis are reinforced by the findings observed in the thyroidectomized-low-protein group (TL).

The alterations promoted by the low-protein diet (L) disappeared when the protein supply was restituted by diet. After an initial period of increased aorta lipid content ($L \rightarrow S_2$) and total cholesterol in plasma, all these measured parameters returned to control values ($L \rightarrow S_4$), with the exception of aortic hydroxyproline levels, that, although increased, remained lower than control; these facts have also been demonstrated (5).

When the refeeding was made together with thyroidectomy, the increase in plasma total cholesterol was stronger ($L \rightarrow T_2$), the same occurring with the aorta lipid content. These values remained high at the fourth week of recovering ($L \rightarrow T_4$). Thyroidectomy also impaired the return of the protein content in aorta and plasma to control levels and produced a stronger decrease in hydroxyproline levels, probably by the same reasons stated above.

The increase in the lipid levels promoted by refeeding in the thyroidectomized rats were not evident, probably by the elevated lipid levels promoted by thyroidectomy itself. In other words, although refeeding promoted an increase in protein levels in aorta and plasma, it was a slight increase, indicating that the recovery, at least in this period of time, was inhibited by thyroidectomy. Then, it seems that a normal thyroid function is important in the recovery of the undernutrition, at least in relation to the observed data.

This could be easily demonstrated by the administration of T_3 to thyroidectomized rats during the recovery period, when all the measured parameters, again with the exception of hydroxyproline, probably by the lower turnover rate, returned to control levels at the end of the experimental period ($TL \rightarrow [T_3]_4$).

In conclusion, 1) it seems that the physiologically active levels of T_3 could contribute for the lower aorta lipid levels observed in low-protein fed rats, with all evidence being consistent with a hyperthyroid response, and 2) the recovery of a normal aorta metabolism after a protein undernutrition period is not possible without a normal thyroid function.

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REFERENCES

1. Levy, R., Rifkind, B., Dennis, B., Ernst, N. Nutrition, lipids and coronary heart disease. (Raven Press, New York, 1979).
2. Srinivasan, S.R., Radhakrishnamurthy, B., Dalferes, E.R., Webber L.S., Berenson, G.S. Serum lipoprotein responses to exogenous cholesterol in spider monkeys: Effect of levels of dietary protein. *Proc. Soc. Exp. Biol. Med.* 154, 102-106 (1977).
3. Bydlowski, S.P., Stivaletti, V.L.G., Douglas, C.R. Influence of dietary cholesterol on lipid, hexosamine, and hydroxyproline content of the arterial wall of undernourished rats. *IRCS Med. Sci.* 9, 743-744 (1981).
4. Bydlowski, S.P., Stivaletti, V.L.G., Tarasantchi, J., Douglas, C.R. Free fatty acids and total and free cholesterol concentration in plasma and arterial wall of undernourished rats under influence of dietary cholesterol excess. *IRCS Med. Sci.* 9, 895 (1981).
5. Bydlowski, S.P., Stivaletti, V.L.G., Douglas, C.R. Biochemical observations on rat aorta: Effect of protein refeeding after a protein depletion period. *Ann. Nutr. Metab.* 28, 85-91 (1984).
6. Sawaya, A.L., Lunn, P.G. Evidence suggesting that the elevated plasma triiodothyronine concentration of rats fed on protein deficient diet is physiologically active. *Br. J. Nutr.* 53, 175-181 (1985).
7. Bydlowski, S.P., Stivaletti, V.L.G., Douglas, C.R. Effect of a low protein-high lipid diet on plasma lipid levels of rats. *IRCS Med. Sci.* 9, 834 (1981).
8. Rosenthal, H.L., Pfluke, B.S.M.L., Buscaglia, S. A stable iron reagent for determination of cholesterol. *J. Lab. Clin. Med.* 50, 318-322 (1957).
9. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275, (1951).
10. Fitch, S.M., Harkness, M.L.R., Harkness, R.D. Extraction of collagen from tissues. *Nature, Lond.* 176, 163 (1955).
11. Folch, J., Lees, M., Stanley, G.H.S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497-509 (1957).
12. Deykin, D., Goodman, D.S. The hydrolysis of long-chain fatty acids esters of cholesterol with rat liver enzymes. *J. Biol. Chem.* 237, 3649-3651 (1962).
13. Baginski, E.S., Foa, P.P., Zak, B. Microdetermination of inorganic

phosphate, phospholipids and total phosphate in biologic materials. Clin. Chem. 13, 326-332 (1967).

14. Carlson, L.A. Determination of serum triglycerides. J. Atheroscler. Res. 3, 334-336 (1963).
15. Neuman, R.E., Logan, M.A. The determination of hydroxyproline. J. Biol. Chem. 184, 299-306 (1950).
16. Mathe, D., Chevallier, F. Effects of the thyroid state on cholesterol metabolism in the rat. Biochim. Biophys. Acta 441, 155-164 (1976).
17. Stivaletti, V.L.G., Douglas, C.R., Bydlowski, S.P. Thyroidectomy-promoting changes on lipid and protein content from aorta of low-protein-fed rats. IRCS Med. Sci. 9, 1119-1120 (1981).
18. Lunn, P.G., Austin, S. Excess energy intake promotes development of hypoalbuminemia in rats fed on low-protein diets. Br. J. Nutr. 49, 9-15 (1983).

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HAIR TRACE ELEMENT CONCENTRATIONS IN PATIENTS WITH PROTEIN-ENERGY MALNUTRITION

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ABSTRACT

The changes in hair trace elements (zinc, copper, chromium, iron, manganese) in 48 protein-energy malnutrition (PEM) cases of different degrees of severity were investigated. Hair trace element concentrations were determined by the flameless atomic absorption technique. Hair zinc levels were relatively low in the PEM cases of all degrees of severity, but the difference from control subjects were not of statistical significance. Low hair zinc concentration (less than 70 $\mu\text{g/g}$) was more frequent in patients with PEM than in normal infants. Hair copper concentrations for all PEM subjects and controls were nearly identical. Although mean hair chromium levels in PEM cases were higher than the controls, no significant differences were observed between these values. Hair iron and manganese concentrations were significantly high in PEM cases. The results of this study indicate that, with the exception of PEM cases who have low hair trace element concentrations, hair cannot be considered to be a reliable biological material in the assessment of trace element nutritional state in malnourished infants.

INTRODUCTION

Hair constitutes a biological material for trace element analysis because of its relatively high concentration of metals and because of its convenience in handling and sampling. However, there are many difficulties in the interpretation of results of hair analyses (1). One of these problems arises from the rate of hair growth. Hair growth rates are depressed or arrested in severe malnutrition, but are probably unaffected in mild and/or moderate forms of malnutrition. Therefore, clear cut interpretation of hair trace element analyses in patients with protein-energy malnutrition is difficult.

Reported results on zinc, copper and chromium concentrations of hair in malnourished infants vary widely (2-7). Iron and manganese concentrations of hair in protein-energy malnutrition has, to our knowledge, not been investigated.

This present study was designed to assess the changes in hair trace elements zinc, copper, chromium, iron, manganese in protein-energy malnutrition cases of different degrees of severity.

MATERIAL AND METHODS

Forty-eight malnourished Turkish infants and children, aged 4 to 30 months (13.02 ± 6.1) (mean \pm SD) were studied. Twenty-five of these patients were classified as mild and/or moderate and twenty-three as severe PEM, using weight/height/age criteria proposed by McLaren and Read (8). All severe PEM cases in the series were marasmic in type according to the Wellcome criteria (9). Twenty-nine normal Turkish infants and children of ages 1 to 36 months attending a well-baby clinic served as controls.

Consent was obtained from the parents and the study had the approval of the Dean of the Istanbul Faculty of Medicine.

Hair samples were analyzed for zinc, copper, chromium, iron, and manganese. These samples were taken from the suboccipital area of the head in immediate proximity to the scalp and stored in plastic bags. Collections were not limited to the occipital area in cases of PEM when there was a sparsity of hair. Prior to the collection of hair samples, it was insured that subjects were not using any hair shampoo or coloring substance.

Trace element concentrations were determined by the flameless atomic absorption technique using a Perkin-Elmer 503 double-beam atomic absorption spectrometer equipped with both a HGA-2100 graphite furnace and model 56 recorder.

Duplicate hair samples of approximately 50 to 100 mg were washed sequentially in hexane, analytical grade ethanol, and deionized distilled water (3 times). They were dried at 110°C in a vacuum oven overnight, weighed and then placed in a low temperature asher (Trapelo LTA 505, LFE Waltham, MA) where they were ashed for 2h (1 mm O_2 pressure, RF power 400 watts forward, 3 to 5 watts reflected). The ashed samples were then treated with 500 μl of 1N HCl, redried and reashed for 1 additional hour. Samples were then dissolved in 500 μl of 1N HCl and 50 μl aliquots injected into the graphite furnace for analysis.

The instrumental parameters used for the analysis of each element are presented in Table 1. Working standards for trace elements were prepared daily from certified atomic absorption standards (Fisher Scientific Co.). The validity of analytical methods was verified by the use of NBS Bovine Liver, Standard Reference Material No. 1577; Washinton, D.C.

Student's t test and chi square test were used to compare the various malnourished groups with the control group. Statistical significance was accepted at the 5% level.

Table 1. Instrumental parameters

Element	Perkin-Elmer 503			HGA-2100		
	Wave-length nm	SBW nm	Drying ^a T-sec	Charring T-sec	Atomization T-sec	Gas flow ^b cc / min
Zn	213.9	0.7	100-30	450-30	2000-7	230
Cu	324.7	0.7	100-30	700-30	2500-7	40
Cr	357.8	0.7	100-20	1100-30	2700-7	40
Fe	248.3	0.2	100-30	1100-30	2700-7	40
Mn	280.3	0.2	100-20	1100-30	2500-7	40

^aT=Temperature °C ^bArgon

RESULTS AND DISCUSSION

Table 2 shows hair trace element (zinc, copper, chromium, iron, manganese) concentrations in the PEM cases and in control subjects.

Table 2. Mean hair trace element levels in the different forms of PEM cases and control subjects

Hair trace element levels
($\mu\text{g/g}$)

Groups	Zn	Cu	Cr	Fe	Mn
mild and/or moderate PEM (25)	134 \pm 116*	14 \pm 4	0.670 \pm 0.355	32 \pm 13	1.076 \pm 0.610
Severe PEM (marasmus) (23)	128 \pm 101	17 \pm 10	0.628 \pm 0.276	31 \pm 11	1.296 \pm 0.918
Control subjects (29)	162 \pm 76	17 \pm 5	0.590 \pm 0.322	25 \pm 7	0.774 \pm 0.406

* mean \pm SD

Hair Fe conc: Mild and/or moderate PEM versus controls, $t=2.3941$, $p<0.02$; severe PEM versus controls, $t=2.1554$, $p<0.05$.

Hair Mn conc: Mild and/or moderate PEM versus controls, $t=2.1687$, $p<0.05$; severe PEM versus controls, $t=2.7487$, $p<0.01$.

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Mean hair zinc concentration of control subjects was $162 \pm 76 \mu\text{g/g}$ (mean \pm SD). Hair zinc levels were relatively low in the PEM cases of all degrees of severity, but the differences from control subjects were not of statistical significance. Neither was any significant difference noted between mild-moderate or severe PEM cases in hair zinc concentration. Hambidge et al (10) reported low hair zinc values in mild to moderate malnutrition. On the other hand, normal and even abnormally high hair zinc levels have been reported in severely malnourished subjects (3,5,11). When the results for each PEM cases were analyzed individually, hair zinc levels were found lower than the values corresponding to -1 SD ($86 \mu\text{g}$ zinc/g hair) in normal controls in twelve of 25 infants with mild and/or moderate PEM, and 11 of 23 severe PEM cases. Low hair zinc levels were noted also in four of the 29 control subjects. However, the proportion of subjects with low hair zinc was significantly higher in the PEM group compared to the control subjects ($\chi^2=7.5431$ $p<0.01$ for mild and/or moderate PEM; $\chi^2=7.2472$ $p<0.01$ for severe PEM). Seven of 25 infants with mild to moderate PEM, and 9 of 23 severe PEM cases also had values of less than $70 \mu\text{g}$ of zinc per gram of hair, the generally accepted lower limit of normal range (2). No children had hair zinc levels less than $70 \mu\text{g/g}$ in control subjects. In a survey of children in Denver, low hair zinc levels were found to be associated with growth failure (2). The incidence of low hair zinc levels was also found to be much higher among preschool children with low growth percentiles from low-income families (10).

Mean copper concentration in hair for all PEM subjects and controls were nearly identical in our series. This is in agreement with the results reported by Gopalan et al (6) who found normal hair copper concentration in children with marasmus, in contrast to the low values encountered in kwashiorkor cases.

Mean chromium levels in hair were $0.590 \pm 0.322 \mu\text{g/g}$, $0.670 \pm 0.355 \mu\text{g/g}$ and $0.628 \pm 0.276 \mu\text{g/g}$ for controls, mild and/or moderate forms of PEM and severe PEM cases. Although mean hair chromium levels in PEM cases were higher than the controls, no significant differences were observed between these values. Chromium deficiency occurs in association with protein-energy malnutrition in some areas of the world, specifically Jordan, Nigeria and Turkey (12,13). However, hair chromium concentrations in the underweight group of children and in the protein calorie malnutrition cases as well as in the "poorly nourished" adult subjects did not deviate from the values obtained on normal children and well nourished adults (7).

Mean hair iron concentrations in this series were significantly high in mild and/or moderate and severe PEM cases

as compared to control subjects ($t=2$, 3941, $p<0.02$; $t=2$, 1554, $p<0.05$). No difference was noted between mild-moderate or severe PEM cases in hair iron concentration.

Mean hair manganese levels in the different degrees of PEM infants were also significantly high as compared to the control subjects ($t=2$, 1687, $p<0.05$; $t=2$, 7487, $p<0.01$). No significant differences in hair manganese concentrations were observed in the different groups of PEM cases. The explanation for these high hair iron and manganese concentration is possibly related to decreased hair growth in mild-moderate or severe cases of malnutrition.

Our results show that 1) Low hair zinc concentration is more frequent in patients with PEM malnutrition than in normal infants, although there is no statistical difference in mean hair zinc between malnourished infants and controls, 2) Hair iron and manganese concentrations are significantly high in PEM cases.

In conclusion it may be stated that, with the exception of PEM cases who have low hair trace element concentrations, hair trace element concentrations cannot be considered to be a reliable indicator in the assessment of trace element nutritional state in malnourished infants. Studies relating concentration of trace elements in hair to rate of hair growth might be helpful in interpretation of the results of these analyses

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REFERENCES

1. Hambidge, K.M. Hair Analyses: Worthless for Vitamins, Limited for Minerals. *Am. J. Clin. Nutr.* 36, 943 (1982).
2. Hambidge, K.M., Hambidge, C., Jacobs, M. and Baum, J.D. Low Levels of Zinc in Hair, Anorexia, Poor Growth and Hypogeusia in Children. *Pediat. Res.* 6, 868 (1972).
3. Bradfield, R.B., Yee, T. and Baertl, J.M. Hair Zinc Levels of Andean Indian Children During Protein-Calorie Malnutrition. *Am. J. Clin. Nutr.* 22, 1349 (1969).
4. McBean, L.D. Mahloudji, M.S., Reinhold, J.G. and Halsted, J.A. Correlation of Zinc Concentrations in Human Plasma and Hair. *Am. J. Clin. Nutr.* 22, 506 (1971).

5. Erten, J., Arcasoy, A., Cavdar, A.O. and Cin, S. Hair Zinc Levels in Healthy and Malnourished Children. *Am. J. Clin. Nutr.* 31, 1172 (1978).
6. Gopalan, C., Reddy, V. and Mohan, V.S. Some Aspects of Copper Metabolism in Protein-Calorie Malnutrition. *J. Pediat.* 63, 646 (1963).
7. Gürson, C.T., Saner, G., Mertz, W., Wolf, W.R. and Sökdü, S. Nutritional Significance of Chromium in Different Chronological Age Groups and in Populations Differing in Nutritional Backgrounds. *Nutr. Rep. Int.* 12, 9 (1975).
8. McLaren, D.S. and Read, W.W.C. Classification of Nutritional Status in Early Childhood. *Lancet* ii, 146 (1972).
9. Classification of Infantile Malnutrition. *Lancet* ii, 302 (1970).
10. Hambidge, K.M., Walravens, P.A., Brown, R.M., Webster, J., White, S., Anthony, M. and Roth, M.L. Zinc Nutrition of Preschool Children in the Denver Head Start Program. *Am. J. Clin. Nutr.* 29, 734 (1976).
11. Hambidge, K.M. Zinc Deficiency in Children. In: Trace Element Metabolism in Animals-2 (W.G. Hoekstra, J.W. Suttie, H.E. Ganther and W. Mertz, editors) University Park Press, Baltimore, MD 1974.
12. Hopkins, L.L., Ransome-Kuti, O. and Majaj, A.S. Improvement of Impaired Carbohydrate Metabolism by Chromium (III) in Malnourished Infants. *Am. J. Clin. Nutr.* 21, 203 (1968).
13. Gürson, C.T. and Saner, G. Effect of Chromium on Glucose Utilization in Marasmic Protein-Calorie Malnutrition. *Am. J. Clin. Nutr.* 24, 1313 (1971).

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BIOLOGICAL AVAILABILITY OF MINERALS IN TROPICAL FEEDSTUFFS FOR GUINEA FOWLS (Numida meleagris galeata, Pallas)

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ABSTRACT

A rapid assay was conducted to determine the biological availability of minerals (Ca, P, Mg, Mn) in groundnut cake, palm kernel meal, maize, cassava meal, rice bran, fish meal, dried distiller's grain and brewer's yeast for mature guinea fowls. Data on mineral availability showed significant differences among the feedstuffs tested. Availability for Ca was 55.7 to 85.7%, P was 28.6 to 88.8%, Mg was 62.1 to 85.8% and Mn was 63.4 to 87.6%. Results indicate that calcium is well utilized in most feedstuffs except maize. Phosphorus in maize was poorly available but well retained from other feedstuffs tested. The availability of magnesium and manganese was high and showed less variability between feedstuffs.

INTRODUCTION

There are indications (Vogt and Stute, 1974) that the efficiency of the guinea fowl to utilize nutrients differs significantly from that of the chicken. Guinea fowls, therefore, may have nutrient requirements different from those of the chicken. Attempts to establish requirements for the guinea fowl must be matched with research efforts to document the biological availability of nutrients from various feedstuffs. The concept of biological availability implies that the availability of nutrients from different feedstuffs does vary and that those differences in availability can be quantified and, therefore, the sources can be compared.

The study reported here was conducted to establish the biological availability for the guinea fowl, of calcium, phosphorus magnesium and managanese found in some tropical feedstuffs.

MATERIALS AND METHODS

Eight commonly used tropical feedstuffs, groundnut cake (GNC), palm kernel meal (PKM), maize (Mz), cassava meal (CM), rice bran (RB), fish meal (FM),

dried distiller's grain (DDG) and brewer's yeast (BY), were each fed to male guinea fowls to determine their retention of calcium, phosphorus, magnesium and manganese. The experimental arrangement was a completely randomized design with four replicates per dietary treatment.

Thirty two adult male guinea fowls were randomly shared into eight groups of four birds each. The four birds within each group constituting four replicates were individually housed in stainless steel metabolism cages. Prior to the start of the experiment, birds were maintained on a 16% crude protein diet. Water containing adequate level of vitamins was freely provided during the experiment. A synthetic diet was formulated to consist of sucrose (81.07%), cellulose (8.8%) and vegetable oil (10.13%). The procedure is a modification of that described by Nwokolo et al. (1976).

On the first day of the trial, birds were allowed 8 hr to consume the maintenance mash into which was incorporated 1.0% inert ferric oxide marker, starved for 24 hr, allowed 8 hr to consume the synthetic diet and returned to the marked mash. Faeces from the synthetic diet was collected and represented all faeces from the end of the first batch of marked excreta to the beginning of the second batch of marked excreta. The feeding procedure was repeated except that the test diets (Table 3) replaced the synthetic diet. Faeces corresponding to the test diets were collected in-between the marked faeces. Faecal collections were oven-dried at 60°C for 48 hr. All test feedstuffs, the test diets, faecal collections from synthetic and test diets were analysed for calcium, phosphorus, magnesium and manganese. The determination of calcium, magnesium and manganese were conducted by atomic absorption spectrometry following wet ashing with perchloric and nitric acids (Johnson and Ulrich, 1959). Phosphorus was determined with spectro-photometer following colour development with ammonium molybdate.

The formula of Nwokolo et al. (1976) was used to estimate per cent mineral availability as follows:

$$\frac{TMI - (IFME - EFME)}{TMI} \times \frac{100}{1}$$

where TMI = Total mineral intake from ingredient
(test feed)

IFME = Ingredient faecal mineral excreted

EFME = Endogenous faecal mineral excreted
(purified diet excreta).

Analysis of variance was performed on the data according to Steel and Torrie (1980) and differences among means were separated by Duncan's multiple range test.

RESULTS

The chemical composition of the test feedstuffs is shown in Table 1. With the exception of maize, cassava and rice bran, the feedstuffs tested were high protein feedstuffs. Table 2 shows the mineral contents of the individual feedstuffs. Except the fish meal which had calcium content of 4.98, other feedstuffs had less than one per cent calcium. Phosphorus content was low for all except rice bran, fish meal and brewer's yeast with 1.41%, 2.5% and 1.3% respectively. Table 4 shows the per cent availability of the minerals in the feedstuffs tested.

Table 1. Proximate composition of test feedstuffs

Feedstuff	%, ON DRY MATTER BASIS					
	DM (%)	CP	CF	EE	Ash	NFE
GNC	94.99	45.19	5.10	8.65	1.92	34.13
PKM	93.55	17.00	9.30	6.10	15.60	45.0
Maize	93.17	9.20	1.35	3.41	0.98	78.23
Cassava meal	94.97	1.9	4.10	0.80	2.10	91.80
Rice bran	90.8	12.4	11.6	13.6	13.3	39.90
Fish meal	94.1	56.8	0.90	11.10	24.40	0.90
DDG	92.3	24.4	11.5	5.60	2.50	48.30
Brewer's yeast	94.0	44.90	2.70	0.70	6.90	38.80

DISCUSSION

Calcium. The results show that calcium from the various feedstuffs is readily available although in varying degrees. Fish meal, groundnut cake and cassava with respective availability values of 91.3%, 85.69% and 80.08%, provided the best utilized calcium. Maize, rice bran, dried distiller's grain and brewer's yeast, all of which provided between 55 and 61% available

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Table 2. Mineral Content of Feedstuffs

Feedstuff	Ca	%		Mn (mg/kg)
		P	Mg	
GNC	0.194	0.625	0.043	100.00
PKM	0.348	0.803	0.163	62.50
Maize	0.016	0.278	0.235	12.50
CM	0.085	0.108	0.014	8.10
Rice bran	0.068	1.410	1.010	298.4
Fish meal	4.980	2.510	0.180	27.50
DDG	0.210	0.800	0.150	28.10
Brewer's yeast	0.140	1.310	0.250	5.20

Table 3. The Composition of test diets

Ingredients	Diets							
	GNC	PKM	MZ	CM	RB	FM	DDG	BY
Groundnut cake	750g	-	-	-	-	-	-	-
Palm kernel meal	-	750g	-	-	-	-	-	-
Maize	-	-	750g	-	-	-	-	-
Cassava meal	-	-	-	750g	-	-	-	-
Rice bran	-	-	-	-	750g	-	-	-
Fish meal	-	-	-	-	-	750g	-	-
Dried distiller's grain	-	-	-	-	-	-	750g	-
Brewer's yeast	-	-	-	-	-	-	-	750g
Synthetic diet	750g	750g	750g	750g	750g	750g	750g	750g

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Table 4. Per cent availability of minerals in test feedstuffs

Feedstuff	Availability %			
	Ca	P	Mg	Mn
Groundnut cake	85.69 ^b	72.01 ^{bc}	85.77 ^a	81.19 ^b
Palm kernel cake	80.46 ^c	69.17 ^c	67.24 ^c	64.35 ^{cd}
Maize	55.69 ^e	28.57 ^e	62.09 ^d	87.64 ^a
Cassava	80.08 ^c	63.59 ^d	66.51 ^c	61.47 ^d
Rice bran	61.24 ^d	73.69 ^{bc}	79.87 ^b	68.72 ^c
Fish meal	91.30 ^a	88.32 ^a	78.46 ^b	81.74 ^b
Dried distiller's grain	60.71 ^d	69.98 ^c	78.89 ^b	63.44 ^d
Brewer's yeast	60.71 ^d	75.55 ^b	77.81 ^b	68.93 ^c

a, b, c, d, e ... Values in each column having different superscripts are significantly different (P/0.05).

calcium can, relative to other test feedstuffs, be described as of intermediate values. The calcium availability value of 85.69% observed for GNC in this trial is similar to that (85.6%) reported for soybean meal by Nwokolo et al. (1976). However, the value of 80.5% we observed for PKM is higher than 64.6% availability reported for the same feedstuff by Nwokolo et al. (1976). The difference may be due in part to the fact that broiler chicks were used by Nwokolo et al., (1976) while mature male guinea fowls were used in this study. The observed difference may also be due to differences in source of PKM and the age of the birds used. The relatively low calcium availability observed for maize may be due to the high phytate content of maize which has been shown to reduce the availability of calcium (Nelson, 1967).

Phosphorus. With the exception of maize, phosphorus availability values for the feedstuffs studied are high and range between 63.6 and 88.3%. The low availability value for maize confirm the observation of

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Temperton and Cassidy (1964) that maize has high phytate content that is highly unavailable. Earlier reports by McCance and Widdowson (1944) and Mollgaard (1946) show that while wheat, rye and barley are high in phytase activity, oats, maize and various seed meals contain little or none of the enzyme. The low phosphorus availability from maize can, therefore, be ascribed to its lack of the phytase enzyme. Phosphorus availability of 69.2% observed for PKM in this study is similar to the value of 70.8% reported by Nwokolo et al. (1976) for PKM. Following high phosphorus availability values, Salman and McGinnis (1968) were led to conclude that hens could utilize a substantial proportion of the phosphorus in plant materials. It appears from the current results that the Guinea fowl is capable of utilizing a high proportion of phosphorus in plant materials. Nelson (1967) summarized from available data that the utilization of phytate phosphorus increases with increasing age to maturity. The high availability of phosphorus from most feedstuffs studied may be due in part to the age of birds used in this study.

Magnesium. Availability of magnesium in all test feedstuffs is high and range between 62 and 65.8%. Although GNC gave the highest availability value, its actual content of magnesium is low. Thus GNC contributes very little to the requirement of the birds. Rice bran is high in magnesium and with 79.9% availability, can supply most of the animals requirement. The magnesium availability in PKM (67.2%) is higher than the value of 56.4% reported for broiler chicks by Nwokolo et al. (1976).

Manganese. Manganese was highly available in all the feedstuffs tested. The highest values were observed for GNC (81.2%), maize (87.6%) and FM (81.7%). Nwokolo et al. (1976) reported manganese availability of 45.7% for PKM. In this study 64.4% availability was observed. This variation as earlier indicated may be due to age and species differences. It is worthy of note that rice bran is particularly high in manganese (298.4 ppm). Thus with an availability value of 68.7%, rice bran can be expected to fulfil the birds' requirement if included in the diet.

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REFERENCES

- Johnson, C.M. and A. Ulrich. 1959. II: Analytical Methods for use in plant analysis. Bull. 766. California Agr. Exp. Sta., Berkeley.
- McCance, R.A., and E.M. Widdowson. 1944. Activity of the phytase in different cereals and its resistance to dry heat. Nature, 153: 650.
- Mollgaard, H. 1946. On phytic acid, its importance in metabolism and its enzymic cleavage in bread supplemented with calcium. Biochem. J. 40:589-603.
- Nelson, T.S. 1967. The utilization of phytate phosphorus by poultry - A review. Poultry Sci. 46: 862-871.
- Nwokolo, E.N., D.B. Brag and W.D. Kitts. 1976. A method for estimating the mineral availability in feedstuffs. Poultry Sci. 55: 2217-2221.
- Salman, A.J. and J. McGinnis. 1968. Availability of phosphorus from plant origin for layers. Poultry Sci. 47: 1712 (abstr.).
- Steel, R.G.D. and J.H. Torrie. 1980. Principles and procedures of statistics. McGraw-Hill Book Co. N.Y.
- Temperton, H. and J. Cassidy. 1964. Phosphorus requirements of poultry. 1. The utilization of phytin phosphorus by the chick as indicated by balance experiments. Br. Poultry Sci. 5:75-80.
- Vogt, H. and A. Stute. 1974. Digestibility of some carbohydrate fractions in hens and guinea fowls. Archir. fur. Gefiugekunde 38(4): 117-118.

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WEIGHT GAIN AND DIGESTIVE TRACT DEVELOPMENT AT 42 DAYS IN LAMBS WEANED TO A DRY DIET AT 10, 14, OR 28 DAYS OF AGE

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ABSTRACT

Thirty-two Finnish Landrace lambs removed from their dams at one day of age were assigned randomly at three days of age to four treatment groups. Lambs were penned individually in .3 x 1.2 m wire-bottom cages and were weaned from liquid milk replacer to a dry modified pig starter diet at 10, 14 or 28 days of age. Half of the lambs weaned at 10 days of age received tap water (500 ml) twice daily from a nursing bottle; all lambs had free access to tap water flowing continuously through a plastic trough attached to all pens. Body weight and dry and liquid feed consumption of each lamb were recorded at 0, 10, 28 and 42 days. Sixteen lambs (4/treatment group) were used to monitor blood hemoglobin, hematocrit and plasma total protein, albumin, urea-N, glucose and acetate weekly throughout the 42 day experiment. At slaughter, the gastrointestinal tract, kidney and liver were removed from each lamb; pH of rumen, abomasum and colon and full and empty weights of rumen-reticulum, abomasum, cecum and small intestine were recorded and samples of rumen and cecum contents were saved for determination of volatile fatty acid concentrations. Daily weight gain to 28 days was greater in lambs weaned to dry diet at 28 days (DD28) than in other lambs ($P<.01$), but from 28 to 42 days the reverse was true ($P<.01$). Overall daily gain (0 to 42 days) was greater in DD28 lambs than in lambs weaned at 10 or 14 days ($P<.01$). Gain/feed was unaffected by treatment during the periods 10 to 28 days and 29 to 42 days, but was greater from 0 to 42 days for DD28 lambs than for those weaned earlier. Hemoglobin, hematocrit ($P<.01$) and plasma glucose ($P<.05$) were higher and plasma urea-N was lower ($P<.01$) in DD28 lambs than in other lambs. There was no effect of treatment on plasma total protein, albumin or acetate. Full and empty weights of rumen-reticulum, abomasum and cecum and concentrations of volatile fatty acids in rumen and cecum at 42 days were unaffected by treatment. It is concluded that the lamb weaned to a dry diet at 10 to 14 days of age is a fully functioning ruminant by day 42 and that weaning at 10 days is associated with survival and growth at least equal to values obtained when weaning is at 14 days. Weight gain to 42 days may be increased by weaning at 28 days rather than at 10 or 14 days.

INTRODUCTION

The use of liquid milk replacers to rear lambs whose dams are unable to provide adequate maternal care is practiced commercially in flocks with high prolificacy. Lambs reared artificially on liquid milk gain weight normally [1,2]. The high cost of labor, equipment and milk

replacer limit the economic feasibility of this practice. A dry feed, patterned after baby pig feed, has been used successfully to rear lambs weaned from liquid milk replacer at 10 days of age [3,4].

The purpose of the present experiment was to determine the survival, weight gain, feed utilization and gastrointestinal tract response of lambs weaned to a dry diet at 10, 14, or 28 days of age.

EXPERIMENTAL PROCEDURE

Thirty-two Finnish Landrace newborn lambs (12 to 24 hr old) were brought to a temperature-controlled room, given 50 ml bovine colostrum by stomach tube and trained during the first day to suckle from a nursing bottle containing a liquid synthetic milk replacer. Lambs were selected from litters of 3 or 4 (2 lambs left with each ewe). Lambs were penned singly in .3 x 1.2 m raised wooden pens with steel mesh floors. Lambs were assigned at about three days of age sequentially to the following four experimental treatments: (1) weaned to dry feed at 28 days (DD28); (2) weaned to dry feed (Table I) at 10 days (DD10); (3) weaned to dry feed at 14 days (DD14); and (4) weaned to dry feed at 10 days and given 500 ml tap water twice daily through a nursing bottle from 10 to 28 days. Initial weight of all lambs was $2.55 \pm .18$ kg. Males were left intact and all lambs were docked during week 1. Individual body weights were recorded on entry into the pens (day 0), initially (day of assignment to treatment, day 1), and on days 10, 14, 17, 21, 28, 35, and 42. Feed intake (liquid plus dry feed) was recorded daily and dry matter intakes from day 0 to 10, 11 to 28, and 28 to 42

Table I. Composition of Dry Diet for Early-weaned Lambs
(Fed in Meal Form)

Ingredient	Percentage
Oats, finely ground (IFN 4-03-315)	10.0
Dried whey product (IFN 4-01-186)	5.0
Alfalfa meal (IFN 1-00-023)	10.0
Corn, No. 2 yellow dent (IFN 4-02-931)	12.2
Dextrose	15.0
Hydrogenated vegetable oil ^a	5.0
Soybean meal (IFN 5-04-604)	40.0
Dicalcium phosphate (IFN 6-01-080)	1.5
Iodized salt (IFN 6-04-151)	.5
Trace mineral premix F ^b	.4
Vitamin premix No. 7 ^c	.2
Choline chloride	.2
Total	100.0

^a Crisco; Proctor and Gamble & Co., Cincinnati, OH.

^b Swine trace mineral premix; contains 75% ground limestone as a carrier; supplies the following (ppm in complete diet): CuO, 10; FeSO₄·7H₂O, 160; MnO, 20; ZnO, 100.

^c Swine vitamin premix. Supplies the following (units/kg of complete diet): vitamin A, 5,280 IU; vitamin D₃, 704 IU; vitamin E, 70.4 IU; vitamin K, 3.52 mg; vitamin B₁₂, 26.4 µg; riboflavin, 5 mg; niacin, 28 mg; d-pantothenic acid, 21 mg; biotin, 88 µg; thiamin, 2.2 mg.

and for the total experiment (day 0 to 42) were computed and used to compute efficiency of feed utilization (gain:feed ratio) during each time interval. Liquid milk replacer was prepared by mixing 250 g of dry LAMAL (Carnation Company, Milling Division, Los Angeles, CA 90036) with 1 liter of tap water and fed ad libitum without heating.

Blood was sampled from the jugular vein of four lambs fed each diet on days 0 and 3 and at weekly intervals thereafter to 42 days for determination of hemoglobin [5], hematocrit, total plasma protein [6], albumin, glucose [7], urea-N [7] and acetate [7]. At 42 days all surviving lambs among those from which blood had been sampled were euthanized with phenobarbital and liver, kidneys and total gastrointestinal tract were removed. Weights of liver, kidneys and of full and empty rumen-reticulum, small intestine, abomasum and cecum were recorded. The pH values of rumen and colon contents were recorded and samples of strained fluid from each organ were frozen at -80C until analyzed for volatile fatty acid concentrations by gas chromatography with a flame ionization detector (Hewlett Packard 5840A Gas Chromatograph).

All data were subjected to least-squares analysis of variance [8]. The effect of sex was in the original model but it had no effect on any trait measured and was therefore deleted. Main effects were diet and time; effects of diet and time and interactions were tested.

RESULTS AND DISCUSSION

Effects of diet treatment on daily gain, dry matter consumption and gain:feed ratio are summarized in Table II. Daily weight gain, dry matter intake and gain:feed ratio during the first 10 days when all lambs received liquid milk replacer ad libitum were not significantly different among treatment groups. From 10 to 28 days DD28 lambs had greater daily weight gain and dry matter intake than other lambs; gain:feed ratio was not significantly affected by diet, although the trend was for higher efficiency in DD28 lambs than in other lambs. Daily gain from day 29 to 42 was less ($P<.01$) in DD28 lambs than in lambs weaned to dry feed earlier, but mean daily dry matter consumed was not significantly affected by treatment. From day 0 to 42, DD28 lambs gained weight more rapidly than lambs weaned earlier ($P<.01$), consumed more dry matter ($P<.01$) and had a higher gain:feed ratio ($P<.01$) than other lambs. The superior performance of lambs weaned at 28 days compared with that of lambs weaned at 10 or 14 days in the present experiment (Figure 1) differs from a previous report [4] in which daily weight gains were similar in lambs weaned to a dry diet at 10 or 28 days. Daily gains to 42 days of age averaged only 100 to 130 grams in those experiments compared with 129 to 195 grams in the present experiment. Unquantified environmental effects present in the current work compared with the previous report [4] may account for these discrepant results. The previous experiments were performed in a large lamb nursery which accommodated several hundred lambs at once, whereas the present experiment was done in a clean room not previously or concurrently occupied by other lambs. Sanitation and probably a lower subclinical disease level in the present experiment may have favored superior lamb performance. In the previous work, voluntary intake of liquid milk replacer may have been curtailed in lambs continued on that treatment to 28 days because

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Table II. Effect of Age at Weaning to a Dry Diet on Weight Gain, Dry Matter Intake and Gain:Feed in Lambs (Least-Squares Means)^a

Diets	DD10	DD10H ₂ O	DD14	DD28 ^b	SD	Prob- ability
No. of lambs	8	8	8	8		
Survivors to 42 days	7 ^c	3 ^d	7 ^e	8		
Daily weight gain, g						
0 - 10 days	169	151	149	170	47	.70
11 - 28 days	62	95	90	226	61	<0.01
29 - 42 days	182	184	208	167	62	<0.01
0 - 42 days	129	145	152	195	28	<0.01
Dry matter consumed						
0 - 10 days	179	178	167	183	36	.79
11 - 28 days	157	158	159	253	56	<0.01
29 - 42 days	326	315	367	286	77	.32
0 - 42 days	216	209	238	264	38	.12
Gain/Feed						
0 - 10 days	.938	.829	.916	.919	.164	.52
11 - 28 days	.269	.739	.488	.767	.390	.11
29 - 42 days	.585	.565	.587	.539	.167	.94
0 - 42 days	.629	.697	.660	.741	.046	<0.01

^a No effect of sex was detected; therefore, data for males and females were combined.

^b DD = dry diet (see table I for composition).

^c One lamb died at 23 days of age.

^d Lambs died or were removed from the experiment 19, 19, 20, 21, and 21 days of age.

^e One lamb died at 27 days of age.

the capacity of the milk dispenser was insufficient to insure that milk was available at all times, whereas in the present experiment special care was taken to assure continuous access to liquid milk in DD28 lambs. Dry matter (including that from liquid milk replacer plus that obtained from DD) consumed by DD28 lambs in the present experiment was 4554 g from day 10 to 28, but only about 3600 g in the corresponding period in the previous report [4].

Blood data are summarized in Table III. Hemoglobin and hematocrit of DD10, DD10H₂O and DD14 lambs were lower ($P < .01$) than values for DD28 lambs, suggesting suboptimal intake or utilization of Fe from the dry diet. The mineral premix included in the dry diet provided 160 ppm of Fe to the complete diet. The quantitative dietary Fe requirement of the suckling age lamb is not clearly established [9] but would be expected to approximate that of other young mammals, including the pig whose requirement at 5 to 10 kg body weight is 140 ppm [10]. Whether the lower daily weight gain of lambs weaned to the dry diet at less than 28 days was causally related to their lower hemoglobin and hematocrit can-

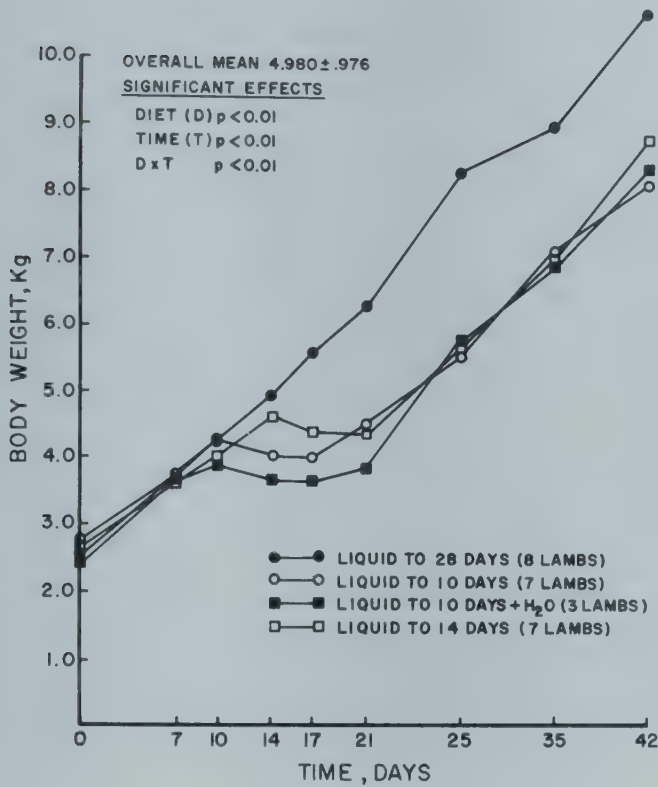


Figure 1. Body weight to 42 days of Finnish Landrace lambs weaned to a dry diet at 10, 14 or 28 days of age (least-squares means).

not be ascertained from the present data. There appears to be a need to establish more clearly the Fe requirement of early-weaned lambs.

Plasma urea-N was greater ($P < .05$) for DD10, DD10H₂O and DD14 lambs than for DD28 lambs, reflecting either less efficient utilization of the N present in the dry diet or an excess of dietary N in relation to needs for net protein accretion [11]. Total plasma protein and albumin were unaffected by dietary treatment, indicating that dietary protein adequacy was not a limiting factor in lamb growth. Plasma acetate concentration was similar for all groups throughout the experiment. The overall mean concentration declined from 36.8 micromoles/ml at day 0 to 24.7, 19.3 and 14.2 micromoles/ml at days 7, 28 and 42, respectively. The relatively high values at days 0 and 7 probably reflected the ingestion of 30 percent fat in the liquid milk replacer fed to all lambs, while the values at 28 and 42 days were probably associated with production of volatile fatty acids from rumen fermentation. The similar plasma acetate concentrations at 28 and 42 days of lambs weaned to the dry diet at 28, 10 and 14 days suggests that development of rumen function was not impaired by any of the diet treatments. The reason for the higher plasma acetate concentrations observed in lambs in this experiment than values reported in mature sheep [12] is unknown. Plasma glucose rose sharply in all groups from day 0 to 3 (81.4 vs 139.6 mg/dl)

Table III. Effect of Age at Weaning to a Dry Diet on Blood Traits in Lambs (Least-Squares Means)^a

	Diets(D):	DD10	DD10H ₂ O	DD14	DD28 ^b	SD	Prob- ability
Number of lambs		4	4 ^c	4	4		
Hematocrit, % RBC		35.7	37.2	37.9	40.3	4.9	T<.01, D<.01
Hemoglobin, g/dl		12.1	12.8	12.8	13.6	1.6	T<.01, D<.01
Plasma urea-N, mg/dl		18.9	18.8	15.7	13.2	7.7	T<.01, D<.05
Plasma glucose, mg/dl		112.5	106.5	108.1	119.5	18.9	T<.01, DxT<.01
Plasma total protein, mg/dl		5.5	5.2	5.5	5.1	.71	T<.01, DxT<.05
Plasma albumin, mg/dl		3.5	3.5	3.6	3.7	.25	T<.01
Plasma acetate, m/ml		21.4	21.1	22.8	25.0	7.77	T<.01

^a Each value is the mean of 32 observations (4 lambs each sampled at 0, 3, 7, 14, 21, 28, 35, and 42 days).

^b DD = dry diet (see Table I for composition); T = Time, D = Diet.

^c One of four lambs died after 21 days; therefore, the mean includes only values for three lambs at days 28, 35 and 42.

and then plateaued to day 21 in DD28 lambs, but in lambs fed other diets the peak reached at day 3 was maintained only until day 7 and then tended to decline to day 21 (values at day 21 were 138.5, 106.6, 88.3 and 102.3 mg/dl for DD28, DD10, DD10H₂O and DD14 lambs, respectively). By day 35, values for all four diet groups were similar (106.9, 100.3, 95.6 and 100.2 mg/dl, respectively) and remained similar at day 42. Overall mean plasma glucose for DD28 lambs tended to be greater than for other lambs but the effect was not significant. There was an overall decline with time (P<.01); there was a significant diet x time interaction (P<.01).

Data on organ weights and on pH of gastrointestinal tract contents at 42 days are summarized on Table IV. Diet had no effect on relative kidney or liver weight, full or empty rumen-reticulum, abomasum, cecum or small intestine weight (percent of live body weight) or pH of rumen, abomasum or colon contents. The absence of differences in full or empty gastrointestinal organ weights and in pH of contents indicates that age at weaning to a dry diet had no influence on physical development or digestive function in lambs slaughtered at 6 weeks of age. Concentrations of volatile fatty acids in rumen and cecum contents (Table V) at 42 days also were not affected by age at weaning, suggesting no long term adverse effects on rumen or lower gastrointestinal tract fermentation associated with early weaning in lambs. Previous work [4] indicated that immature physical or physiological traits associated with gastrointestinal function may be related to rumen impaction and higher mortality in lambs weaned at 10 days of age com-

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Table IV. Effect of Age at Weaning to a Dry Diet on Organ Weights and on pH of Gastrointestinal Segments in Lambs at 42 Days of Age (Least-Squares Means)

Diets(D):	DD10	DD10H ₂ O	DD14	DD28	SD	Prob- ability
Number of lambs	4	3	4	4		
Kidney wt, % of wt.	.072	.059	.081	.062	.015	.63
Liver wt, % of wt.	.283	.236	.295	.233	.030	.24
Full rumen-reticulum wt, % of b wt.	1.52	1.15	1.28	1.19	.31	.69
Empty rumen-reticulum wt, % of b. wt.	.27	.26	.26	.20	.03	.18
Full abomasum wt, % of b. wt.	.35	.38	.37	.37	.115	.29
Empty abomasum wt, % of b. wt.	.083	.091	.090	.077	.007	.13
Full cecum wt, % of b. wt.	.303	.342	.298	.264	.048	.72
Empty cecum wt, % of b. wt.	.135	.158	.136	.117	.018	.47
Full sm. intestine wt, % of b. wt.	.495	.473	.525	.494	.070	.78
Empty sm. intestine wt, % of b. wt.	.370	.337	.391	.330	.062	.70
Rumen pH	6.64	6.28	6.55	6.46	.27	.52
Abomasum pH	3.47	3.29	3.11	2.94	.54	.79
Colon pH	6.70	6.58	6.61	6.61	.14	.85

Table V. Effect of Age at Weaning to a Dry Diet on Gastrointestinal Tract Volatile Fatty Acid Concentrations in Lambs at 42 Days of Age (Least-Squares Means)(Micromoles/ml)

Diets:	DD10	DD10H ₂ O	DD14	DD28	SD	Prob- ability
Number of lambs	4	3	4	4		
Rumen						
Acetate	23.86	25.79	35.18	31.39	8.55	.59
Propionate	9.21	10.61	20.79	13.44	6.08	.27
Isobutyrate	0.52	0.93	0.68	0.43	.25	.39
Butyrate	8.87	13.92	11.58	13.13	4.70	.67
Valerate	2.55	4.39	4.27	3.93	1.37	.65
Caproate	0.73	1.27	1.54	1.37	.65	.38
Isovalerate	0.61	1.44	1.22	0.56	.27	.09
Cecum						
Acetate	83.50	110.49	102.43	93.73	17.20	.33
Propionate	17.33	20.00	26.31	18.42	4.28	.17
Isobutyrate	1.69	2.50	1.89	1.50	.73	.57
Butyrate	8.39	10.93	11.69	10.05	3.16	.76
Valerate	1.35	1.76	1.53	1.34	1.86	.91
Isovalerate	4.71	6.37	4.72	4.73	.97	.63

pared with results in lambs weaned later. In the present experiment no death losses were attributed to rumen impaction and only in DD10H₂O lambs was there an indication of a relationship between diet treatment and death. In this group, five of the eight lambs died or were removed between 19 and 21 days of age. These losses were associated with low intake of dry diet and a gradual decline in vigor and weight gain ending in death. In two cases, lambs found comatose in their pens were quickly revived by intravenous glucose administration. They were removed from the experiment and returned to the liquid milk replacer diet until vigor was regained. The better acceptance of transfer to the dry diet by DD10 and DD14 lambs than by DD10H₂O lambs suggests that access to extra water may have been detrimental to a higher survival rate. The provision of supplemental water from a nursing bottle had no apparent benefit to performance in a previous comparison [4]. There is no clear evidence from the results of the present experiment that physical or physiological immaturity of the gastrointestinal tract of lambs weaned to a dry diet at 10 days of age is an important factor in lamb survival. The data support the conclusion of Lane and Hogue [13] that the lamb weaned to a dry diet at 14 days of age rapidly becomes functionally a ruminant. Further work is needed to determine the cause of the greater dry matter intake of lambs fed a liquid milk replacer diet to 28 days than of lambs weaned to a dry diet at 10 days of age and the consequent weight gain advantage. The limitations and adaptations of the gastrointestinal tract of lambs in response to age and diet [14,15,16,17,18,19,20,21] need further clarification by serial gastrointestinal and metabolic measurements during growth of lambs weaned at various ages between birth and 28 days.

It is concluded that the lamb weaned to a dry diet at 10 or 14 days of age is a fully functioning ruminant by day 42 and that weaning at 10 days of age is associated with growth and survival at least equal to values obtained when weaning is at 14 days. Weight gain to 42 days may be increased by weaning at 28 days rather than at 10 or 14 days.

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Mention of a trade name, proprietary product or specific experiment does not constitute a guarantee and(or) endorsement by the USDA and does not imply its approval to the exclusion of other similar products that may be suitable.

REFERENCES

1. Lindahl, I.L., Sidwell, G.M. and Terrill, C.E. Performance of artificially reared Finnsheep-cross lambs. *J. Anim. Sci.* 34, 935 (1972).
2. Fredericksen, K.R., Jordan, R.M. and Terrell, C.E. Rearing lambs

NUTRITION REPORTS INTERNATIONAL

- on milk repacer diets. USDA Farmers' Bulletin No. 2270, 22 (1980).
3. Magee, B.H. The weaning of lambs to dry feed at 10 days of age. M.S. Thesis. Cornell University, Ithaca, NY (1978).
 4. Pond, W.G., Ferrell, C.L., Jenkins, T.G. and Young, L.D. Weaning of lambs to a dry diet at ten days of age. J. Anim. Sci. 55, 1284 (1982).
 5. Sanford, H. and Sheard, C. A photoelectric hemoglobinometer. J. Lab. Clin. Med. 14, 558 (1929).
 6. Gornall, A.G., Bardawill, C.J. and David, M.M. Determination of serum protein by means of the biuret reaction. J. Biol. Chem 177, 751 (1949).
 7. Gilford Diagnostics. Gilford Diagnostics, Cleveland, Ohio, pp 1-7 (based on method of Talke, H. and Shubert, G.E.; Klin. Waehn. 43, 1965 (1978).
 8. SAS User's Guide. Statistical Analysis Systems Institute, Inc., Cary, NC (1979).
 9. NCR. Nutrient Requirements of Sheep. National Research Council Publications No. 5. National Academy of Sciences, Washington, DC (1975).
 10. NCR. Nutrient Requirements of Swine. National Research Council Publication No. 2. National Academy of Sciences, Washington, DC (1979).
 11. Eggum, B.O. Blood urea measurement as a technique for assessing protein quality. British J. Nutr. 24, 983 (1972).
 12. Ross, J.P. and Kitts, W.D. Relationship between postprandial plasma volatile fatty acids, glucose and insulin levels in sheep fed different feeds. J. Nutr. 103, 488 (1973).
 13. Lane, S.F. and Hogue, D.E. Metabolic changes in lambs weaned at 14 days of age. Proc. Cornell Nutr. Conf. pp 34-41 (1983).
 14. Walker, D.M. The development of the digestive enzyme system of the young animal. III. Carbohydrase enzyme development in the young lamb. J. Agr. Sci. (Camb.) 53, 374 (1959).
 15. Walker, D.M. The development of the digestive enzyme system of the young animal. IV. Proteolytic enzyme development in the young lamb. J. Agr. Sci. (Camb) 53, 381 (1959).
 16. Wardrop, I.D. and Coombe, J.B. The post-natal growth of the visceral organs of the lamb. I. The growth of the visceral organs of the grazing lamb from birth to sixteen weeks of age. J. Agr. Sci. (Camb.) 54, 140 (1960).

NUTRITION REPORTS INTERNATIONAL

17. Wardrop, I.D. and Coombe, J.B. The development of rumen function in the lamb. *Australian J. Agr. Res.* 12, 661 (1961).
18. Walker, D.M. and Walker, G.J. The development of the digestive system of the young animal. V. The development of rumen function in the young lamb. *J. Agr. Sci. (Camb.)* 57, 271 (1961).
19. Church, D.C., Jessup, G.L. Jr. and Bogart, R. Stomach development in the suckling lamb. *Amer. J. Vet. Res.* 23, 220 (1962).
20. Stephens, D.B. and Baldwin, B.A. Observations on the behaviour of groups of artificially reared lambs. *Res. Vet. Sci.* 12, 219 (1971).
21. Szegedi, B., Juhos, B., Szelenyi-Galantai, M., Jecsai, J. and Teleki, M. Experimental studies on early-weaned lambs. *Hungarian Acta Vet. Acad. Sci.* 28, 71 (1980).

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THE EFFECT OF YEAST CULTURE ON THE POSTSTRESS PERFORMANCE OF FEEDER CALVES.

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ABSTRACT

Steer and heifer beef calves averaging less than 8 months old and 208 kg were subjected to weaning, fasting, refeeding and fasting a second time in order to simulate the sequence of events found in marketing channels which move feeder calves from the farm of origin to the next production point. These events resulted in changes in body weight similar to those observed in the actual marketing environment and poststress dry matter intake (DMI) was depressed by about 50% of maximum intake. Dry matter intake increased over time ($P < .01$) in all trials and peaked at 3 weeks poststress. Yeast culture was added to the poststress diet at 1% or 2% of the dry matter to study its effect on dry matter intake and poststress performance. Dry matter intake tended to be increased with the addition of yeast culture, but no differences were observed between 1 and 2% yeast culture. Weight gain was not consistently increased by the addition of yeast culture to the receiving diet. Dry matter intake during the first week was never below 1.4% of body weight, thus the effect of yeast culture in an extremely depressed animal was not tested. Adding monensin to the poststress diet decreased DMI by 9%, but when the depression in DMI was corrected by adding yeast culture to the diet, performance was ($P < .01$) reduced. Thus the addition of yeast culture did not consistently increase poststress performance.

INTRODUCTION

Annual losses due to Bovine Respiratory Disease (BRD) have been estimated to exceed 500 million (1). The stresses associated with weaning, marketing and transit have been reviewed by Phillips (2) and result in a decrease in the calf's natural resistance to the etiologic agents involved in BRD. During these stressful events energy intake is low and body reserves must be mobilized to meet the nutrient needs of the calf. Replenishing these body reserves and meeting the elevated posttransit needs are further complicated by a depression in dry matter intake (3). Lofgreen et al. (4) compensated for the lower dry matter intake by increasing the energy level of the receiving diet. Increasing poststress dry matter consumption increased nutrient intake and animal performance. The ability of the calf to maintain resistance to infectious diseases also depends on nutritional status. Natural feed additives such as yeast have been shown to increase feed intake, average daily gain and feed efficiency in unstressed ruminants but results have not been consistent (5). Yeast products may be effective in increasing poststress dry matter intake of beef calves. The objective of

the following studies was to determine the effect of adding yeast culture to the poststress diet of feeder calves on feed intake and performance.

MATERIALS AND METHODS

Three trials using 144 freshly weaned calves were conducted over a 12 month period beginning in October, 1983. A brief description of each trial is presented in table 1.

Table 1. Description of the calves used in each trial.

Trial	Date	Sex ^a	Breed	No. of Calves	Weaning Weight	Percent Shrink	Poststress Weight
1A	10-83	S	X bred	23	191 ^b	11	170 ^b
1B	6-84	H	X bred	18	213	7	198
2	10-84	S	X bred	56	235	9	215
3	10-84	S	Hereford and X bred	47	-	-	165

^aS = steers, H = heifers; ^bkilograms.

The calves used in Trials 1A and 1B were born and reared on the Livestock and Forage Research Laboratory, El Reno, Oklahoma and were approximately 8 months old when they were subjected to the following sequence of events to simulate the stress of marketing and transit; weaned and fasted for 24 h (auction barn phase), fed medium quality wheat hay for 72 h (order buyer phase), and fasted again for 36 h (transportation phase). After the stress period calves were randomly allotted to one of three pens equipped with pinpointers units^{ab} to measure individual feed consumption. One of the first three experimental diets shown in table 2 were fed to each pen. The control diet was similar to the receiving diet previously used at this location (6). The two yeast diets contained a commercial yeast culture which had been grown in a corn based medium then dried^{ac}. Yeast culture was added to the control diet at 1 or 2% of the dry matter and at the expense of corn and cottonseed hulls. All diets were formulated to be isonitrogenous (11.9% crude protein), isocaloric (1.95 Mcal NEm per kg and 1.1 Mcal per NEg kg), contain 1.5% potassium and were fed ad libitum for 28 d.

^aMention of trade name or product does not constitute a recommendation or endorsement for use by the USDA.

^bQUIS Corporation, P.O. Box 951, Cookeville, TN 38501.

^cYeast culture, Diamond V Mills, Inc., P.O. Box 4408, Cedar Rapids, IA 52451

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Table 2. Composition of experimental diets.

Ingredient ^a	Control	1% Yeast	2% Yeast	Monensin	Yeast and Monensin ^b
Corn	53.8	53.3	52.8	53.7	53.2
Cottonseed hulls	35.1	34.6	34.1	35.2	34.7
Soybean meal	7.9	7.9	7.9	7.9	7.9
Molasses	.9	.9	.9	.9	.9
Urea	.3	.3	.3	.3	.3
Yeast culture	--	1.0	2.0	--	1.0
Calcium sulfate	.6	.6	.6	.6	.6
Calcium carbonate	.1	.1	.1	.1	.1
Potassium chloride	1.3	1.3	1.3	1.3	1.3

^aPercent of dry matter.

^b27 grams per metric ton.

Dry matter intake (DMI) and changes in body weight were measured weekly with a final weight preceded by a 16 h fast taken on day 29 to determine gain in body mass. Samples of diets were dried at 65 C for 72 h to determine dry matter content. Data from trial 1 were analyzed as a split plot in time with main plots being replication (1A and 1B) and diet using pens as experimental units. Differences among means were determined by the Honest Significant Difference (HSD) procedure if a significant F value was observed in the analysis of variance (7).

Steer calves used in trial 2 were subjected to the same sequence of events as used in trial 1, but the transit event was different. After 17 h of fasting during the transit phase one half of the calves were transported in a livestock trailer for 12 h then fasted for another 12 h. The other half were continuously fasted for the 41 h transit period. After the transit period the calves within each group were randomly allotted to six pens. Three pens formed a replicate and each of the three diets fed in trial 1 were fed in one pen in each replicate. Dry matter intake and body weight changes were measured as described in trial 1. Data were analyzed as a split plot in time with a factorial arrangement of treatments (3 diets x trucked or fasted). Differences among means were determined by HSD procedure.

Calves used in trial 3 had been weaned and assembled for 2 days prior to being transported 130 km to the research facilities. Upon arrival each calf was weighed and randomly assigned to one of eight pens, four pens per block. Pens were 7.6 x 15.2 m, open dirt lots with fence line bunk feeders. Treatments, randomly assigned to pens within a block, were 0 or 1% yeast culture with and without monensin^d. Observations were made on a pen basis at weekly intervals. Data were

^dElanco Products Company, 740 S. Alabama St., Indianapolis, IN 46285.

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analyzed as a split plot in time with a factorial arrangements of treatments. Differences among means were determined by the HSD procedure, if a significant F value was observed (7).

RESULTS AND DISCUSSION

Changes in body weight as result of the stressful events imposed were similar for trials 1A and 1B (table 1) and are comparable to values reported for calves that were stressed in a simulated environment or assembled and transported through normal marketing channels (3, 4, 8, 9, 10, 11). Heifers used in trial 1B did not lose as much weight as steers used in trial 1A. This was probably due to the fact that the heifers were born in the fall and spent the winter with their dams which were supplemented with hay. Thus when hay was offered during the 72-h order buyer phase the heifers were accustom to hay as a feed source and as a result weighed 104% of their weaning weight before entering the transit phase. Steer calves were born in the spring and were not accustom to consuming hay and regained only 99.7% of their weaning weight during the order buyer phase. Similar observations have been made in previous trials (Phillips, unpublished data).

Calves which received a diet containing yeast culture tended ($P < .10$) to consume more dry matter than the calves fed the control diet when dry matter intake (DMI) was averaged over the 28-d test period (table 3). Adding yeast culture to the diet did not increase DMI during the first week of the test period, but initial DMI was not depressed below 1.2% of body weight. Dry matter intake did increase ($P < .01$) with time, and peaked by week 3 (1A) and week 2 (1B). Initial DMI was 1.8% and 1.5% of body weight for trials 1A and 1B respectively, but DMI increased to 3.3% by the last week of the trial for both steers and heifers. Thus initial DMI was about 50% of maximum.

Diet had no affect on weekly weight changes. Net average daily gain (ADG) was determined by difference between the poststress weight and a shrunk weight on day 29 to reduce the effects of gastrointestinal fill. During the 28-d period net gain was greater ($P < .01$) for calves fed yeast culture diets as compared to calves fed the control diet (table 3). The differences between trial A and B in this experiment are confounded with season and sex of the calf, but steer calves in trial A gained more ($P < .01$) net weight than the heifer calves in trial 1B. These differences between the two sexes were much greater than previously observed (6). Weekly weight gain calculated from initial shrunk weight and weekly full weight were similar from week to week ($10.8 \text{ kg} \cdot \text{hd}^{-1} \cdot \text{week}^{-1}$), but calves fed yeast culture diets tended to gain more weight than calves fed the control diet. Intestinal fill made up a larger portion of the weight gains of all experimental groups. Thus net gain figures presented in table 3 are the best measures of actual gain in body mass. One heifer in the control group lost weight during the 28 d trial and if that animal is excluded from the data set, the control heifers would then have an ADG of $.61 \text{ kg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$, which is similar to the two groups receiving the yeast diets. Thus during trial 1, addition of yeast culture improved ADG when steers were used, but did not affect ADG when heifers were used.

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Table 3. Dry matter intake (DMI) and average daily gain (ADG) of calves in the three experimental diets during trial 1.

Trial	Diet	No. of Calves	Week				Mean DMI	ADG
			1	2	3	4		
1A	Control	8	2.7 ^a (1.5) ^b	5.7 (3.0)	6.6 (3.4)	6.6 (3.2)	5.4	.76 ^c
	1% Yeast	8	3.7 (2.1)	6.6 (3.4)	6.5 (3.2)	6.8 (3.2)	5.5	1.10
	2% Yeast	7	3.4 (1.9)	7.1 (3.6)	8.5 (4.1)	7.6 (3.4)	6.6	1.06
	Mean		3.3	6.5	7.2	7.0	6.0	
1B	Control	6	3.3 (1.6)	5.6 (2.5)	6.3 (2.8)	7.3 (3.1)	5.6	.32
	1% Yeast	6	2.5 (1.2)	6.0 (2.8)	7.8 (3.4)	8.4 (3.5)	6.2	.61
	2% Yeast	6	3.4 (1.7)	6.1 (2.8)	8.2 (3.6)	8.0 (3.4)	6.4	.53
	Mean		3.1	5.9	7.3	7.9	6.1	

^aKg DMI ·hd⁻¹·d⁻¹.

^bValue in parenthesis are DMI as a percent of body weight.

^cKg of net gain per day.

Changes in body weight during the stress period and during the 28 d poststress period of trial 2 were not different ($P>.10$) between calves that had been fasted only or fasted and transported. Adding yeast culture to the receiving diet increased dry matter intake by 24% (5.1 vs 4.1 kg·hd⁻¹·d⁻¹) over those fed the control diet, but these differences were not significant ($P>.10$). Dry matter intake increased ($P<.01$) with time from 3.1 kg·hd·d⁻¹ initially to 7.1 kg·hd⁻¹·d⁻¹ during week 4 (table 4). These values and patterns of change of DMI are quite similar to those observed in Trial 1. Although not statistically different ($P>.10$) calves fed the 1% yeast culture diet had an ADG of .1 kg more than calves fed the control diet (.87 vs .97 kg·hd⁻¹·d⁻¹).

In trial 3 dry matter consumption increased with time ($P<.01$) and was not ($P>.10$) affected by addition of yeast culture as previously observed. Adding monensin to the diet tended ($P<.10$) to decrease overall dry matter intake by 9% (5.4 vs 4.9 kg·l·hd⁻¹·d⁻¹) and in combination with yeast culture provided the poorest ADG ($P<.05$) of the four experimental groups. Calves fed control, yeast culture alone or monesin alone diets had a mean net ADG of 1.04 kg·hd⁻¹·d⁻¹ while those fed diet containing both yeast culture and monesin had an ADG of .87 kg·hd⁻¹·d⁻¹.

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Dry matter intake at weekly intervals for all three trials are presented in table 4. Although these trials were conducted over a 12 month period, under different climatic conditions and with different feeder calves, DMI were similar among the various trials. The amount of dry matter consumed during the first week of each trial was 1.8%, 1.5%, 1.4% and 2.3%. These values are higher than those observed by Lofgreen et al. (4, 8, 9) and Hutcheson et al. (3) for calves assembled and transported under industry conditions. Dry matter consumption rapidly increased ($P<.01$) during the second and third week and then leveled off by the fourth week as previously observed (6). Thus during the first week, dry matter intake was about 50% of feed intakes at the end of the trials.

Table 4. The dry matter intake (DMI) at weekly intervals during each trial.

Trial	Week				Mean
	1	2	3	4	
1A	3.3 ^a (1.8) ^d	6.5 ^b (3.3)	7.2 ^b (3.6)	7.0 ^b (3.3)	6.0
1B	3.1 ^a (1.5)	5.9 ^b (2.7)	7.4 ^c (3.3)	7.9 ^c (3.3)	6.1
2	3.1 ^a (1.4)	5.1 ^{ab} (2.2)	6.0 ^b (2.5)	7.1 ^b (2.9)	5.3
3	3.9 ^a (2.3)	5.1 ^b (3.0)	6.1 ^c (3.3)	5.4 ^{bc} (2.7)	5.1

a,b,cMeans in the same row with different superscripts differ ($P<.01$).

^dValues in parenthesis are DMI expressed as a percent of body weight.

The average initial weight of calves in the present studies was 208 kg therefore daily maintenance requirements were 4.10 Mcal of net energy. This required 2.1 kg of dry matter, thus DMI during week one was adequate for maintenance plus .3 kg of gain. As long as DMI does not drop below 1.0% of body weight and the diet is a high concentrate diet as used in this experiment, energy intake will be sufficient for maintenance based on values for non-stressed calves. Based on the overall ADG (.9) and daily DMI (6 kg) for the four 28 d trials presented in these studies, ADG should have been 1.3 kg. Thus a stressed calf may have nutrient requirements which are 20-30% greater than the non-stressed calf or digestibility of the diet is lower in stressed calves. These observations are in agreement with those of Hutcheson et al. (3) and Cole et al. (12).

Observations made in these experiments are similar to those of previous experiments using yeast products in non-stressed ruminants (5, 13). Ruf et al. (5) reported an increase in ADG and dry matter intake with the addition of yeast to diets fed to lambs, while Adams et al. (13) reported that yeast had no affect on ADG or DMI of 400 kg steers. The mode of action by which yeast culture positively affects ADG and DMI is not clear, thus situations in which a positive response would be anticipated are not predictable. The addition of yeast culture to ruminant diets has been shown to have no affect on nitrogen retention and little affect on diet digestibility (5, 14).

Changes in ruminal function have shown that addition of yeast culture to the diet did not alter volatile fatty acid concentration or nitrogen metabolism in contrast to well documented effects of monensin on ruminal measurements (15), but yeast culture addition did increase ruminal liquid flow rate but not liquid dilution rate (13). The effects of monensin addition to the diet of non-stressed ruminant are more predictable and consistent. It is well documented that when monensin is added to the diet the amount of acetate produced is decreased while the amount of propionate produced is increased (15). Monensin also decreases DMI by about 13%, but does not affect ADG because of increased metabolic efficiency (15). There are indications that monensin may also increase nitrogen utilization (16). In trial 3 of the present experiment, addition of yeast culture did not increase DMI when added alone, but when fed in combination with monensin the depression in DMI observed when monensin was fed alone was corrected. However ADG was the poorest in this group. A possible explanation is that the yeast culture cancelled the positive affects of monensin by altering ruminal turnover rate or affected the microbial population.

In conclusion, simulating the assembly and transit phases of marketing did result in a loss of weight similar to reported values for calves actually moved through the marketing system. Dry matter consumption during the first week after arrival was about 50% of intake achieved by the third week after arrival. Adding yeast culture to the diet at 1% or 2% of the dry matter did not consistently increase DMI or ADG. When yeast culture was fed with monensin the depression in DMI associated with feeding monensin was overcome. The mode of action by which yeast culture can increase DMI and ADG is not known nor is the inconsistency in response to yeast culture understood. The stressed beef calf probably has a higher nutrient requirement than the non-stressed calf.

REFERENCES

1. McMillan, C. W. Proceedings of Bovine Respiratory Disease Symposium. Amarillo, TX p. 65 (1984).
2. Phillips, W. A. Factors associated with stress in beef cattle. Management of Food Producing Animals Symposium. Purdue Univ. p. 640-655. May (1982).
3. Hutcheson, D. P., Cole, N. A. and McLaren, J. B. Effects of pretransit diets and posttransit potassium levels for feeder calves. J. Anim. Sci. 58, 700 (1984).
4. Lofgreen, G. P., Stinocher, L. H. and Kiesling, H. E. Effects of dietary energy, free choice alfalfa hay and mass medication on calves subjected to marketing stresses. J. Anim. Sci. 50, 590 (1980).
5. Ruf, E. W., Hale, W. H. and Burroughs, W., Observations upon an unidentified factor in feedstuffs stimulatory to cellulose digestion in the rumen and improved liveweight gains in lambs. J. Anim. Sci. 12, 731 (1953).

6. Phillips, W. A. The effect of protein source on the poststress performance of steer and heifer calves. *Nutr. Int.* 30, 853 (1984).
7. Steel, R. G. D. and Torrie, J. H. *Principles and Procedures of Statistics.* McGraw Hill Book Co., Inc., New York (1960).
8. Lofgreen, G. P., Addis, D. G., Dunbar, J. R. and Clark, J. G. Time of processing calves subjected to marketing and shipping stress. *J. Anim. Sci.* 47, 1324 (1978).
9. Lofgreen, G. P., Tayeb, A. E. and Kiesling, H. E. Millet and alfalfa hays alone and in combination with high-energy diet for receiving stressed calves. *J. Anim. Sci.* 52, 959 (1981).
10. Camp, T. H., Stevens, D. G., Stermer, R. A. and Anthony, J. P. Transit factors affecting shrink, shipping fever and subsequent performance of feeder calves. *J. Anim. Sci.* 52, 1219 (1981).
11. Cole, N. A., McLaren, J. B. and Hutcheson, D. P. Influence of preweaning and B-vitamin supplementation of the feedlot receiving diet on calves subjected to marketing and transit stress. *J. Anim. Sci.* 54, 911 (1982).
12. Cole, N. A., Hutcheson, D. P., McLaren, J. B. and Phillips, W. A. Influence of pretransit zeranol implant and receiving diet protein and urea levels on performance of yearling steers. *J. Anim. Sci.* 58, 527 (1984).
13. Adams, D. C., Galyean, M. L., Kiesling, H. E., Wallace, J. D. and Finkner, M. D. Influence of viable yeast culture, sodium bicarbonate and monensin on liquid dilution rate, rumen fermentation and feedlot performance of growing steers and digestibility in lambs. *J. Anim. Sci.* 53, 780 (1981).
14. LeGendre, J. R., Totusek, R. and Gallup, W. D. Effect of live cell yeast on nitrogen retention and digestibility of rations by beef cattle. *J. Anim. Sci.* 16, 671 (1957).
15. Raum, A. P., Cooley, C. O., Potter, E. L., Rathmacker, R. P. and Richardson, L. F. Effects of monensin on feed efficiency of feedlot cattle. *J. Anim. Sci.* 43, 670 (1976).
16. Muntifering, R. B., Theurer, B., Swingle, R. S. and Hale, W. H. Effect of monensin on nitrogen utilization and digestibility of concentrate diets by steers. *J. Anim. Sci.* 50, 930 (1980).

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ACCUMULATION OF DIETARY β -CAROTENE IN THE RAT

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ABSTRACT

Female albino rats were fed a 1% β -carotene-fortified diet for up to 17 weeks; a group of animals fed standard Purina Chow served as controls. At selected time intervals, skin and serum samples were obtained for high performance liquid chromatographic determination of β -carotene. The average β -carotene levels accumulated after the fortified diet had been fed for 14 weeks were 66 ng/ml in serum and 146 ng/g in skin. The results show that the rat is capable of accumulating β -carotene from a dietary source, and, furthermore, that a correlation exists between serum and skin β -carotene levels. Studies of this type should be useful in further evaluation of the role of dietary β -carotene in protection against various types of toxicity and carcinogenesis.

INTRODUCTION

β -Carotene is a naturally occurring pigment present in most of the green and yellow vegetables common in our diets. In addition, β -carotene is used both as a food color additive and as a drug for management of the photosensitivity that occurs in persons having the genetic disorder erythropoietic protoporphyria (1).

Recently, the protective role of dietary β -carotene against chemically and light-induced carcinogenesis has become an area of vigorous investigation in both epidemiological (2-4) and experimental studies (5). Experimental studies of β -carotene effects have been limited to some extent by a lack of appropriate animal models, i.e., models in which dietary β -carotene is accumulated and also in which the toxicological end point of interest is easily observed.

Several species are known to accumulate dietary β -carotene to some degree, and humans are good accumulators (6). It has been reported that rodents do not accumulate β -carotene (6). However, in a more recent study (7), mice were found to accumulate β -carotene to a limited degree when given a β -carotene-fortified diet for extended periods. In this report, we present kinetic data of β -carotene profiles in serum and skin of rats, and suggest this animal as a useful model for studies of the protective effects due to dietary β -carotene. The primary purpose in developing this model was the study of protection against chemically induced cutaneous phototoxicity (8). However, this model and the methodology described here may also be useful in investigations of dietary β -carotene protection against carcinogenesis.

MATERIALS AND METHODS

Materials. Purina rodent chow was obtained from the Ralston Purina Co. β -Carotene beadlets, provided by Hoffmann-LaRoche Inc., were composed of 10% (w/w) β -carotene and also contained fractionated coconut oil, gelatin, sucrose, and modified food starch, with BHT, BHA, and ascorbyl palmitate as antioxidants and methyl and propyl parabens, ascorbic acid, and sodium benzoate as preservatives.

Animals. Albino female rats of the Osborne-Mendel strain, weighing 200-300 g, were obtained from the Food and Drug Administration breeding colony.

Diet Preparation. The 1% β -carotene-fortified diet was prepared from β -carotene beadlets and Purina Chow as described by Mathews-Roth et al. (7). Each batch of the β -carotene-fortified diet was analyzed for β -carotene content. Pellets were ground into a powder, then extracted six times with 40-ml aliquots of low boiling petroleum ether under reflux with vigorous stirring. β -Carotene in the combined extracts was quantitated by measuring the absorption at 455 nm with a Cary 17D spectrophotometer, using a standard curve. Twelve batches of fortified diet were analyzed individually and the β -carotene content averaged $1.02 \pm 0.03\%$ by weight.

Feeding Phase of the Experiment. A total of 60 rats were assigned to four groups of 15 each by using a randomization procedure. Rats of three groups were fed the 1% β -carotene-fortified diet daily ad libitum; rats of the remaining group were fed Purina Chow and served as controls. To obtain an indication of the extent to which the rodents accumulated β -carotene, three animals from one group receiving the 1% β -carotene-fortified diet were killed at each of the selected time intervals (3, 4, 5, 6, and 8 weeks). The remaining two β -carotene-fed groups were killed at 14 and 17 weeks. After the rats were killed, skin from clipped areas of the back and serum samples were collected for β -carotene quantitation; samples were processed and analyzed immediately or, when necessary, were stored frozen for later analysis.

Analysis of Rat Serum. An internal standard of lycopene was added to 1-ml aliquots of serum. The samples were then extracted by using a modification of the method of Henry (9). The organic layer resulting from the extraction was evaporated to dryness under nitrogen and reconstituted in 0.2 ml of the mobile phase used in the determination of β -carotene by high performance liquid chromatography (HPLC).

Analysis of Rat Skin. Approximately 0.5 g of rat skin was thoroughly homogenized in 10 ml of 12% KOH in methanol, using a Polytron homogenizer. The internal standard, lycopene, was added to each homogenate, which was then extracted as described by Lee et al. (10). The resulting organic layer was then evaporated under nitrogen and reconstituted in 0.2 ml of the HPLC mobile phase.

HPLC Analysis. Reconstituted serum and skin extracts were analyzed by HPLC, using instrumentation including a Waters Model 6000A solvent delivery system, Model U6K injector, Model 440 absorbance detector

fitted with a 436 nm filter assembly, and data module integrator. A Waters μ Bondapak C₁₈ column, 3.9 mm X 30 cm, 10 μ m particle size, was eluted at 2 ml/min with acetonitrile-methylene chloride-methanol (70:20:10 v/v) (11).

RESULTS

The results obtained in this study indicate that the rat is capable of accumulating β -carotene from a dietary source to a limited degree. β -Carotene levels observed in the rat were low compared to those observed in good accumulators (6). None of the animals fed a β -carotene diet appeared carotenemic during the course of this study. Successful quantitation of serum, and particularly skin levels, required the use of HPLC methods, both to increase sensitivity and to eliminate background interferences common in the alternative spectrophotometric assay. Figure 1 shows chromatograms of serum and skin extracts from a rat with intermediate levels of β -carotene (49

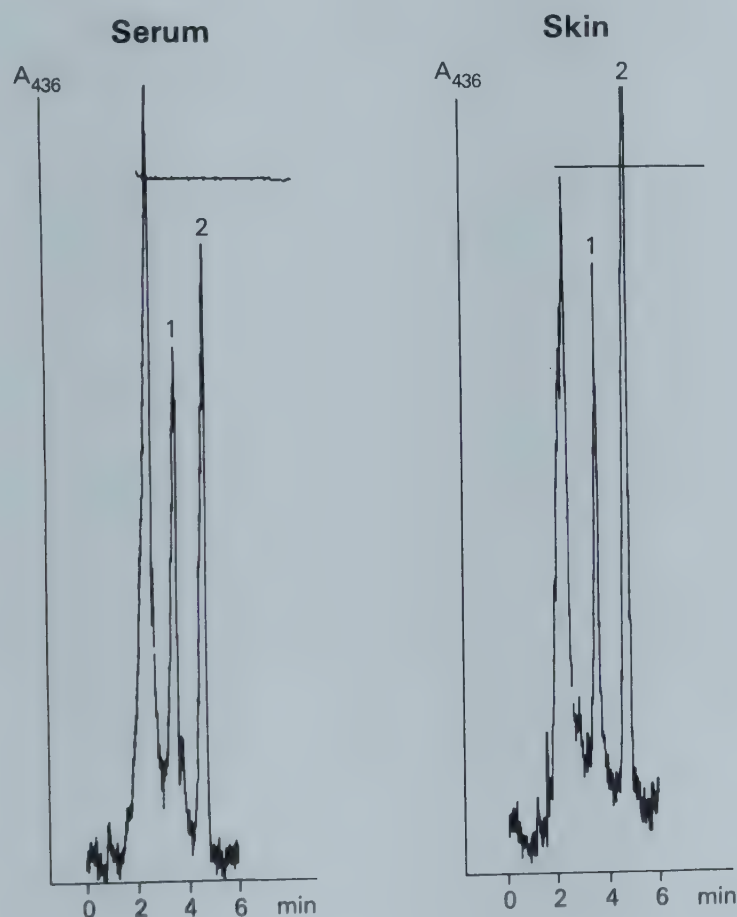


Figure 1. Representative chromatograms obtained in the HPLC determination of β -carotene levels in the serum and skin. The numbered peaks correspond to 1) lycopene, the internal standard, and 2) β -carotene. See Materials and Methods for details of analysis.

Table I. β -Carotene Levels in Serum and Skin of Rats Fed Diets Containing 1% β -Carotene

Weeks on diet	No. of rats	β -Carotene ¹	
		Serum (ng/ml)	Skin (ng/g)
3	3	25.7 + 11.8	46.2 + 13.8
4	3	51.2 + 17.2	95.6 + 19.7
5	3	30.2 + 6.4	73.2 + 7.9
6	3	38.8 + 24.3	88.7 + 41.7
8	3	70.0 + 23.0	109.2 + 30.4
14	15	65.7 + 12.8	145.5 + 15.8
17	15	34.0 + 7.3	134.8 + 22.1

¹Values are means + SEM.

ng/ml and 136 ng/g, respectively). By using this HPLC method, β -carotene levels as low as 10 ng/ml in serum and 20 ng/g in skin can be readily quantitated.

Table I presents the results of the serum and skin analysis for rats killed at selected times within the feeding period. The groups of three rats killed at 3, 4, 5, 6, and 8 weeks served to indicate the extent of β -carotene accumulation early in the study. In spite of the large variations observed, the data suggest that the rats accumulate β -carotene throughout this phase of the feeding period. The data derived from groups of rats killed at 14 and 17 weeks indicate that skin β -carotene levels reach a plateau at these time points. An apparent decrease in serum β -carotene levels was noted at 17 weeks; the reason for this decrease is not known and is currently being investigated. The control serum and skin samples analyzed showed no significant β -carotene content, in agreement with a previous report (12).

In addition to the observed accumulation of β -carotene in serum and skin, we have noted that serum and skin levels correlate well throughout the entire feeding period (Figure 2). These results indicate that the observed β -carotene levels in skin result from distribution of β -carotene in the serum into the skin rather than through topical contamination of the skin.

DISCUSSION

The results presented here indicate that the rat accumulates dietary β -carotene to a limited degree. We have successfully used this animal model to study the protective effect of dietary β -carotene against 8-methoxypsoralen-induced phototoxicity (8). In that study, a clear protective effect was provided by the levels accumulated in skin after the rats had been given the 1% fortified diet for 13 weeks. Thus, although the levels reported here for the rat are low relative to those in species known to be good accumulators, they have biological

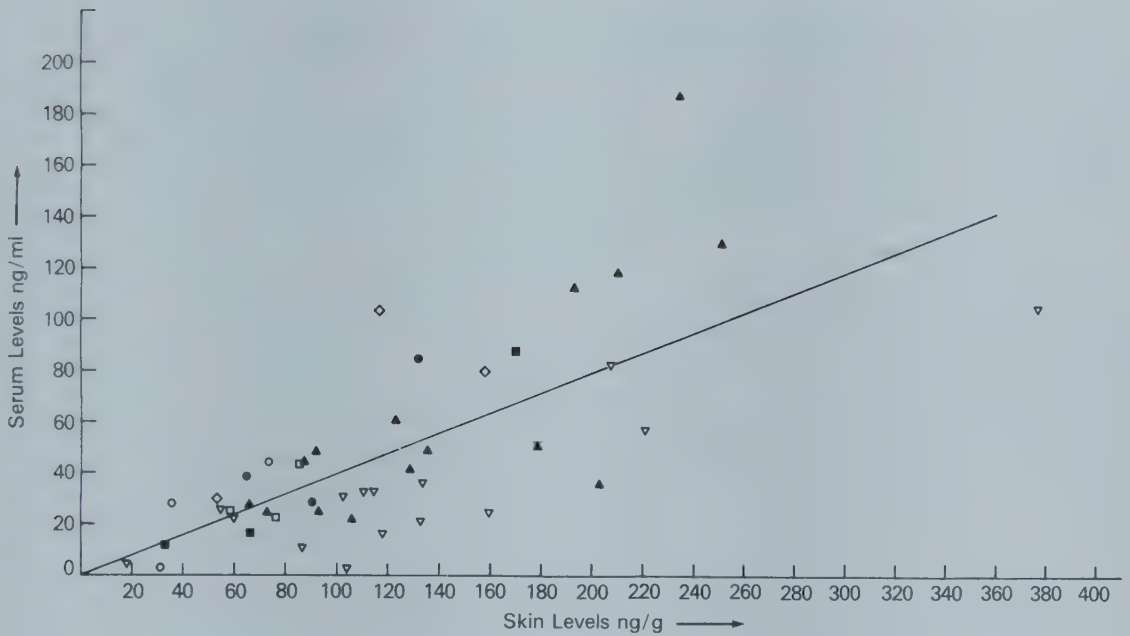


Figure 2. The relationship between serum and skin β -carotene levels for rats on a β -carotene-fortified diet for varying lengths of time. Each point represents data derived from one animal. The following symbols are used to denote the period (weeks) on the fortified diet: 3 (\circ); 4, (\bullet); 5, (\square); 6, (\blacksquare); 8, (\diamond); 14, (\blacktriangle); 17, (∇).

significance. Only skin and serum were analyzed in this study, but it may be reasonably assumed that systemic accumulation results from the dietary regimen used. Indeed, while this manuscript was in preparation, a report of β -carotene accumulation in various organs of the rat appeared (13). Thus, this model may be applicable to studies of the protective effect of β -carotene against toxicity or carcinogenesis in target organs other than skin.

There is reported epidemiological evidence to date to suggest that consumption of certain carotene-rich vegetables is associated with a reduction in the incidence of cancer at several sites in humans (14). Accumulated evidence suggests that β -carotene is responsible for the beneficial effect observed, although a number of other nutritive and nonnutritive components present in these vegetables might modify carcinogenesis in laboratory animals and humans.

The mechanism of protection by β -carotene is still unknown; it could be exerted by its known provitamin A activity or by its action as a natural antioxidant. At least in the case of UVB (290-320 nm)-induced skin neoplasms, an antioxidant type of mechanism has been suggested, whereas a transformation of β -carotene to vitamin A derivatives seems to be the primary mechanism in inhibiting chemical carcinogenesis (5). In any case, the advantage of β -carotene compared with other common antioxidants lies in its extremely low toxicity (4).

Although the majority of experimental and epidemiological studies performed to date suggest a protective effect of carotenoids on cancer (4, 5, 14), a few studies have failed to confirm this (15). Therefore, studies of the type presented in this report are necessary to develop animal models for evaluation of the extent and mechanism of dietary β -carotene protection against carcinogenesis.

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REFERENCES

1. Mathews-Roth, M.M., Pathak, M.A., Fitzpatrick, T.B., Harber, L.C. and Kass, E.H. β -Carotene as an Oral Photoprotective Agent in Erythropoietic Protoporphyria. *J. Am. Med. Assoc.* 228, 1004 (1974).
2. Peto, R., Doll, R., Buckley, J.D. and Sporn, M.B. Can Dietary Beta-Carotene Materially Reduce Human Cancer Rates? *Nature* 290, 201 (1981).
3. Ames, B.N. Dietary Carcinogens and Anticarcinogens. *Science* 221, 1256 (1983).
4. Willett, W.C. and MacMahon, B. Diet and Cancer - An Overview. *N. Engl. J. Med.* 310, 633 (1984).
5. Mathews-Roth, M.M. Antitumor Activity of β -Carotene and Canthaxanthin. *Oncology* 39, 33 (1982).
6. Deuel, H.J. *The Lipids, Their Chemistry and Biochemistry*, Vol. 3. Interscience Press, New York, 1957, p. 424.
7. Mathews-Roth, M.M., Hummel, D. and Crean, C. The Carotenoid Content of Various Organs of Animals Administered Large Amounts of Beta-Carotene. *Nutr. Rep. Int.* 16, 419 (1977).
8. Giles, A., Wamer, W., Kornhauser, A. and Noble, J. In Vivo Protection by β -Carotene Against Phototoxicity. 9th International Congress on Photobiology, Philadelphia, PA, Abst. No. MAM-D7 (1984).
9. Henry, R.J. *Clinical Chemistry, Principles and Technics*. Harper and Row, New York, 1965, p. 704.
10. Lee, R., Mathews-Roth, M.M., Pathak, M.A. and Parrish, J.A. The Detection of Carotenoid Pigments in Human Skin. *J. Invest. Dermatol.* 64, 175 (1975).
11. Nells, H.J.C.F. and De Leenheer, A.P. Isocratic Nonaqueous Reversed-Phase Liquid Chromatography of Carotenoids. *Anal. Chem.* 55, 270 (1983).
12. Bröich, C.R., Gerber, L.F. and Erdman, J.W., Jr. Determination of Lycopene α - and β -Carotene and Retinyl Esters in Human Serum by Reversed-Phase High Performance Liquid Chromatography. *Lipids* 18, 253 (1983).
13. Shapiro, S.S., Mott, D.J. and Machlin, L.J. Kinetic Characteristics of β -Carotene Uptake and Depletion in Rat Tissue. *J. Nutr.* 114, 1924 (1984).

14. Committee on Diet, Nutrition and Cancer: National Research Council. Diet, Nutrition, and Cancer. National Academy Press, Washington, DC, 1982.
15. Willet, W., Polk, B., Underwood, B., Stampfer, M., Pressel, S., Rosner, B., Taylor, J., Schneider, K. and Hames, C. Relation of Serum Vitamins A and E and Carotenoids to the Risk of Cancer. N. Engl. J. Med. 310, 430 (1984).

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A SHORT-TERM EFFECT OF SPECIFIC DIETS AND EXERCISE ON METABOLIC RATE AND CORE TEMPERATURE

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ABSTRACT

The short-term effect of diets and exercise on metabolic rate and core temperature was determined in mildly obese and nonobese females. Participation in mild exercise (a brisk walk at 3 mph) significantly affected the subjects' metabolic rate. Carbohydrate-exercise, fat-exercise, and USDG-exercise significantly affected the metabolic rate. All diets when combined with exercise were significant when compared to the same diet without exercise. The caloric level of the foods was more instrumental in determining DIT than their composition. Regarding core temperature, ANOVA revealed significance among treatment groups in all subjects and in pre-post temperature depending on the type of diet. Temperature appeared to be increased when food and exercise were combined. Carbohydrate-exercise and USDG-exercise were significant with fat-exercise approaching significance at the .05 level.

Introduction

A number of research studies conducted on obesity have demonstrated that many people have great difficulty losing weight regardless of caloric intake. Although research on Dietary Induced Thermogenesis (DIT) has looked at the problems of size and composition of caloric ingestion, most investigators have limited their observations to one group of subjects, nonobese or obese. Those researchers who have investigated obese and nonobese individuals (1,2) have used subjects between 19 and 32 years of age. Other researchers have limited their investigations to meals high or low in protein (3), high in carbohydrates (2), or to a balanced meal (4). There seems to be no research to date that has compared a high carbohydrate, high fat, and balanced meal in the same subjects. Furthermore, investigators have not paired obese and nonobese individuals while subjecting them to the same experimental procedures.

Therefore, this study was undertaken because of the inconclusive results on DIT as seen from past research and the need for more knowledge on how the quantity and composition of food along with exercise affect the metabolic rate and the core temperature of individuals. This study also sought to examine a population as yet uninvestigated, moderately obese females who are sedentary and between the ages of 29-62 years of age.

For the purpose of this paper, metabolic rate was defined as the magnitude of heat production and assessed through the measurement of oxygen uptake (5). Dietary Induced Thermogenesis was defined as the production of heat in response to eating (6,7).

Methods

Subjects and Apparatus

Ten sedentary females 29-62 years of age were paired according to age and placed in one of two predetermined groups, "normal" or mildly obese (Table I). Each pair of subjects was tested under all experimental conditions in a random order over a four week period.

The Beckman Metabolic Measurement Cart (MMC) was used to determine the metabolic response to different diets with and without exercise. A YSI tele-thermometer was used to obtain the subject's rectal temperature. The Lange Skinfold Caliper was used to obtain percent fat determinations.

Treatments

One experimental treatment was administered to each pair of subjects on any given day and the entire experimental period lasted six days for each pair. The experimental treatments were: Resting Metabolic Rate (RMR); Fasting Exercise Rate; High Carbohydrate Breakfast along with 30 minutes sitting (rest); High Carbohydrate Breakfast with 30 minutes walk on a level treadmill at 3 mph (exercise); High Fat Breakfast with 30 minutes rest; High Fat Breakfast with 30 minutes exercise; Breakfast following the U.S. Dietary Guidelines (USDG) with 30 minutes exercise.

Analytical Procedures

RMR was taken for 30 minutes on four of the six days, between 5:00 and 6:00 a.m. after 30 minutes rest, and while the subject was in a supine position and in a postabsorptive state. An average of these randomly chosen four days was used as the RMR value. Caloric value of the breakfast was based upon the subject's total energy needs according to the guidelines of Whitney and Hamilton (8). The following estimated values were used to determine the subject's total estimated energy needs: The subject's BMR was added to her activity level and Specific Dynamic Effect (figured as 10% of total BMR and Activity). One-third of the total estimated energy need was used as a caloric value of the breakfast treatments. The Fasting Exercise Rate (FER) was determined by a 30 minute continued recording of VO_2 while the subject walked on a level treadmill at 3 mph in a postabsorptive state. The resting phase of the treatment consisted of the subject sitting for 30 minutes while the exercise phase consisted of the subject walking on a level treadmill at 3 mph. This level of activity was chosen because of the low fitness condition of the subjects.

Test Meals

The composition of the high carbohydrate breakfast was an average of 86% carbohydrates, 3% fat, and 11% protein. The high fat breakfast was composed of an average of 66% fat, 21% carbohydrate and 13% protein. The USDG breakfast was composed of an average of 62% carbohydrates, 26% fat, and 12% protein. The foods used to compose the high carbohydrate breakfast were: orange juice, corn flakes, sugar, whole-wheat bread, jelly and skim milk. High fat foods selected were: egg yolks, butter,

Table I
Descriptive Statistics of Subjects

Nonobese (N = 5)	Age Years	Height cm	Weight kg	Skinfold % fat	Breakfast Calories	Breakfast Kilojoules
Mean	47	163.3	62.31	32.14	644	2696
SD	11.8	13	6.26	4.25	67.7	283
High	58	185.0	68.8	36.15	757	3169
Low	28	150.3	54.67	27.48	574	2403
Range	31	36	15	10	184	770
Standard error	5.28	5.81	2.80	1.90	30.28	127
Obese (N = 5)						
Mean	47	167.6	80.89	38.49	779	3261
SD	11.8	5.4	9.25	5.3	84.7	355
High	61	171.0	93.70	44.31	891	3730
Low	29	158.0	69.40	30.02	663	2775
Range	33	14	25	15	229	959
Standard error	5.28	2.41	4.14	2.37	37.88	159
t observed		.683	3.719*	2.090	2.784*	

*Significant at the .05 level
tcrit = 2.132

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sandwich spread and whole milk. The foods used for the USDG breakfast were a mixture of the previous foods mentioned.

Investigative Procedures

On the first day of the experimental period, each subject was given a background information sheet to complete; height and weight were measured; skinfold measurements were taken; and the fasting exercise metabolic rate (FER) was determined. On days two through six, the subject's weight was taken and the rectal temperature probe was inserted. If the subject was to receive a RMR-30 minute determination (4 out of 6 days), this was done after 30 minutes of rest. The subject-pair then consumed one of the breakfasts (high carbohydrate, high fat, or USDG), after which they rested for an average of 45 minutes before continuing treatment. The remainder of the treatment consisted of 30 minutes sitting or walking on the treadmill while metabolic and temperature data were collected.

Analysis of Data

Metabolic data which were obtained during the experimental treatments were converted to kilocalories and kilojoules following the method of Consolazio et al. (9), and Lamb (10). The statistical analysis performed was a Randomized Complete Block design. ANOVA using two groups by five age-pairs, by pre-post by seven conditions (2x5x2x7 factorial) with repeated measures on the last two factors was computed. The .05 level of significance was used for all research hypotheses testing unless specifically stated. The data were further probed by a set of 12 nonorthogonal contrasts and later four more nonorthogonal contrasts were utilized. The second part of the analysis was an ANOVA with two groups, by five age-pairs, by seven conditions (2x5x7 factorial). The Newman-Keuls Post Hoc test, and four more nonorthogonal contrasts were further employed.

Results and Discussion

Certain diet conditions combined with exercise significantly affected caloric output. Significance was found among the following treatments: resting metabolic rate (RMR) vs. fasting exercise, RMR vs. high carbohydrate exercise, RMR vs. high fat exercise, RMR vs. USDG exercise, high carbohydrate rest vs. high carbohydrate exercise, high fat rest vs. high fat exercise, and all rest vs. all exercise (Table II). No significant differences were found in metabolic rate between the mildly obese and the nonobese as determined by kilocalories or kilojoules. These results agree with Guyton (11) and McArdle et al. (12) by demonstrating that an exercise induced thermogenic effect exists, and at even low levels of exercise (walking 3 mph) a thermogenic effect can take place. If 3 mph can be interpreted as a brisk walk, perhaps even the obese may be receptive to participation in such moderate exercise.

The results of this study provided additional evidence that the thermic effect of a meal is enhanced by exercise (Table III). These findings support the work of other researchers (3,13,14,15,16,17), all of whom agree that exercise has an increased effect on DIT. In this study,

Table II

Calories Contrasts (N = 10)

Contrast Number	Name	* NS
1	RMR vs. high carbohydrate rest	NS
2	RMR vs. high fat rest	NS
3	RMR vs. fasting exercise	*
4	RMR vs. high carbohydrate exercise	*
5	RMR vs. high fat exercise	*
6	RMR vs. USDG exercise	*
7	Fasting exercise vs. high carbohydrate exercise	NS
8	Fasting exercise vs. high fat exercise	NS
9	Fasting exercise vs. USDG exercise	NS
10	High carbohydrate rest vs. high carbohydrate exercise	*
11	High fat rest vs. high fat exercise	*
12	All rest vs. all exercise	*
13	High carbohydrate rest vs. high fat rest	NS
14	High carbohydrate exercise vs. high fat exercise	NS
15	High carbohydrate exercise vs. USDG exercise	NS
16	High fat exercise vs. USDG exercise	NS

* = significant

NS = nonsignificant

 $p < .05$

RMR = Resting Metabolic Rate

the meal when combined with exercise had an increased thermogenic effect over the meal without exercise. No significant differences in kilocalories were found when food combined with exercise was compared to fasting combined with exercise. All diets when combined with exercise were significant when compared to the same diet without exercise. Based upon the number of kilocalories consumed by the subjects in the present study, it appears that exercise alone accounted for the thermic effect (Table II). Nonobese subjects received an average of 644 kcal (2.6 MJ), and the mildly obese consumed an average of 779 kcal (3.1 MJ).

The absence of short term DIT in this study as measured by the rate of metabolism agrees with Bradfield and Jourdan (18) where DIT was not observed below 500 kcal (2 MJ). No DIT also agrees with Glick et al. (19), and Strong et al. (20) although they all overfed their subjects at least 2000+ kcal/day (8 MJ).

Presently there is much controversy concerning the caloric level and the composition of the diet. Miller et al. (3) and Garrow and Hawes (21) indicate that the caloric level of the meal is more important than the composition in demonstrating DIT. However, Danforth (21), Goldman et al. (16), Kaplan and Leveille (1), and Miller and Mumford (3) all agree that nutrient composition is important in demonstrating DIT. Since liquid meals are metabolized at a faster rate than solid meals, DIT will occur earlier following a liquid meal vs. a solid meal. The composition of the meal will also determine the time that the DIT is observed. A high carbohydrate meal will demonstrate DIT much earlier than a high-fat meal

Table III
Results of 30-minute Treatments (N = 10)

	VO ₂ Liters/min	kcal	kJ	ΔE kcal from resting metabolic rate	ΔE kcal from specific food-rest to food-exercise	Final temperature fahrenheit	ΔE temperature from final resting temperature
Resting Metabolic Rate	0.156	22.6	94.6			97.82	
Fasting Exercise	0.709	98.1	410.6	75.5*		99.18	1.36
Carbohydrate Rest	0.221	34.9	146.1	12.3		98.40	0.58
Fat Rest	0.214	30.7	128.5	8.1		98.11	0.29
Carbohydrate Exercise	0.646	97.8	409.4	75.2*	62.9*	99.13	1.31
Fat Exercise	0.786	114.5	479.3	91.9*	83.8*	99.14	1.32
USDG Exercise	0.768	114.9	481.0	92.3*		98.93	1.11

* = Significance ($\bar{p} < .05$)

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since fats take a much longer time to digest and to be metabolized.

In the present study, it appeared that the low caloric level was the dominant factor rather than the composition of food in determining DIT. There was a specific intention of the researchers to feed the subjects 1/3 of the Calories expected to maintain their weight (based on a three meal a day intake).

When referring to core temperature, ANOVA revealed significance among the treatment groups in all subjects as well as significant differences in pre-post temperature. The difference in pre to post temperature further depended upon the specific diet treatment (Table IV). No significant differences appeared between the mildly obese and nonobese in core temperature. Further analysis of the data also revealed a trend toward significance at the .05 level in three contrasts suggesting an exercise effect: (a) high carbohydrate-exercise over resting, (b) USDG-exercise over resting, and (c) all exercise over all rest (Table V). When examined singularly, neither food nor exercise significantly affected core temperature. However, a trend appeared to be demonstrated when food and exercise were combined. This trend was seen in carbohydrate, USDG and fat when combined with exercise. Analysis of the data revealed significance between two treatments: carbohydrate-exercise and USDG-exercise with fat-exercise approaching significance at the .05 level. Although the low level of exercise appeared to be insufficient for an exercise effect to take place, it was more than adequate to demonstrate a significant effect when combined with a meal containing an adequate carbohydrate level.

Table IV

ANOVA for Differences in Temperature (N = 10)

Source	SS	df	MS	F
Weight group	497.83809	1	497.83809	1.47567
Age pairs	754.54407	4	188.63602	.55915
Weight x age pairs	1349.45673	4	337.36441	
Treatments	993.78561	6	165.63094	2.68616*
Treatments x weight	311.27100	6	51.87850	.68376
Treatments x age pairs	1479.85773	24	61.66074	
Treatments x weight x age pairs	1820.94170	24	75.87257	
Pre vs. post (PP)	936.02849	1	936.02849	54.6245*
PP x weight	37.02860	1	37.02860	1.58581
PP x age pairs	68.54274	4	17.13569	
PP x weight x age pairs	93.40001	4	23.35000	
Treatments x PP	959.27113	6	159.87852	6.89308*
Treatments x PP x weight	61.67146	6	10.27858	.37073
Treatments x PP x age pairs	556.65714	24	23.19405	
Treatments x PP x weight x age pairs	665.39999	24	27.72500	

*F ratio significant ($p < .05$)

Table V

Temperature Contrast Trends (N = 10)

Contrast Number	Name	F ratio
1	RMR vs. high carbohydrate rest	.15
2	RMR vs. high fat rest	.22
3	RMR vs. fasting exercise	3.64
4	RMR vs. high carbohydrate exercise	5.08*
5	RMR vs. high fat exercise	3.98
6	RMR vs. USDG exercise	4.77*
7	Fasting exercise vs. high carbohydrate exercise	.12
8	Fasting exercise vs. high fat exercise	.01
9	Fasting exercise vs. USDG exercise	.08
10	High carbohydrate rest vs. high carbohydrate exercise	3.51
11	High fat rest vs. high fat exercise	2.33
12	All rest vs. all exercise	7.35*

* $p < .05$

Fcrit = 4.26

RMR = Resting Metabolic Rate

Conclusions

The results of this study suggest that participation in even mild exercise (brisk walk) can significantly affect the metabolic rate of sedentary mildly obese and nonobese females. When the mildly obese or nonobese female participates in a low level of exercise after eating a meal, the combination of the meal and exercise demonstrates an increased thermogenic effect.

Regarding core temperature, specific foods when combined with exercise have a significant effect over resting temperature. Food or exercise when considered singularly, however, has no significant effect on resting temperature. Core temperature is affected by diets moderately high or high in carbohydrates when combined with exercise. The USDG diet combined with exercise demonstrated results that are as effective as carbohydrate-exercise and fat-exercise. Therefore, the USDG diet which was recommended by the Committee on Nutrition and Human Needs seems adequate for the mildly obese as well as the nonobese.

Because the nonorthogonal temperature contrast analysis depicted a trend toward an exercise effect at the .05 level among 3 contrasts: (a) high carbohydrate-exercise over resting, (b) USDG-exercise over resting, and (c) all exercise over all rest, the researchers suggest that further investigations isolating these factors in their design is warranted.

Additional research is needed to further explore the exercise effect found among the subjects in this study. It is recommended, however, that exercise be based upon a percent of each subject's maximum VO_2 capacity rather than all subjects performing at the same level of intensity.

References

1. Kaplan, M.L., and Leveille, G.A. Calorigenic Response in Obese and Nonobese Women. *American Journal of Clinical Nutrition*, 29, 1108-1113 (1976).
2. Pittet, P., Chappuis, P., Acheson, K., Techtermann, F., and Jequier, E. Thermic Effect of Glucose in Obese Subjects Studied by Direct and Indirect Calorimetry. *British Journal of Nutrition*, 35:281-292 (1976).
3. Miller, D.S., and Mumford, P. Gluttony 1. An Experimental Study of Overeating Low- or High-protein Diets. *American Journal of Clinical Nutrition*, 20, 1212-1222 (1967).
4. Miller, D.S., Mumford, P., and Stock, M.J. Gluttony 2. Thermogenesis in Overeating Man. *American Journal of Clinical Nutrition*, 20:1223-1229 (1967).
5. Astrand, Per-Olaf, and Kaare Rodahl. *Textbook of Work Physiology*. McGraw-Hill Book Co., New York 1977.
6. Garrow, J.S. The Regulation of Energy Expenditure in Man. In: *Recent Advances in Obesity Research Vol. II*, (G. Bray, editor). Newman Ltd., London 1978.
7. Miller, D.S., and Mumford, P. Thermogenesis. In: *Energy Balance in Man*, (D.S. Miller and P. Mumford, editors). Masson et Cie, Paris 1973.
8. Whitney, E.N., and Hamilton, E.M.N. *Understanding Nutrition*, 2nd edition. West Publishing, New York 1981.
9. Consolazio, C.F., Johnson, R.E., and Pecora, L.J. *Psychological Measurements of Metabolic Functions in Man*. McGraw-Hill Book Co., New York 1963.
10. Lamb, David R. *Physiology of Exercise: Responses and Adaptations*. MacMillan Publishing Co., New York 1984.
1. Guyton, Arthur C. *Textbook of Medical Physiology*. W.B. Saunders Co., Philadelphia 1981.
2. McArdle, W.D., Katch, F.I., and Katch, V.L. *Exercise Physiology: Energy, Nutrition, and Human Performance*. Lea and Febiger, Philadelphia 1981.
3. Bradfield, R., Curtis, D.E., and Margen, S. Effect of Activity on Caloric Response of Obese Women. *American Journal of Clinical Nutrition*, 21(10), 1208-1210 (1968).
14. Bray, G.A., Whipp, B.J., and Koyal, S. N. The Acute Effects of Food Intake on Energy Expenditure During Cycle Ergometry. *American Journal of Clinical Nutrition*, 27, 254-259 (1974).

NUTRITION REPORTS INTERNATIONAL

15. Gleeson, M., Brown, J.F., & Waring, J.J. The Effects of Exercise and Exercise Training on Dietary-induced Thermogenesis. The Proceedings of the Nutrition Society, 388A (1979).
16. Goldman, R.F., Haisman, M.F., Bynum, G., Horton, E.S., & Sims, E.A.H. Experimental Obesity in Man: Metabolic Rate in Relation to Dietary Intake. In: Obesity in Perspective, (G. A. Bray, editor). P. Fogarty International Center Series for Preventive Medicine, Vol. II, Part 2. U.S. Government Printing Office, Washington, D.C. 1976.
17. Himms-Hagen, J. (1976). Cellular Thermogenesis. Annual Review of Physiology, 38, 315-351 (1976).
18. Bradfield, R.B., and Jourdan, M.H. Relative Importance of Specific Dynamic Action in Weight-reduction Diets. Lancet, 1, 640 (1973).
19. Glick, Z., Shvartz, Magazanik, A., & Modan, M. Absence of Increased Thermogenesis During Short-term Overfeeding in Normal and Overweight Women. American Journal of Clinical Nutrition, 30, 1026-1035 (1977).
20. Strong, J.A., Shirling, D., and Passmore, R. Some Effects of Overfeeding for Four Days in Man. British Journal of Nutrition, 21:909- 918 (1967).
21. Garrow, J.S., and Hawes, S.F. The Role of Amino Acid Oxidation in Causing Specific Dynamic Action in Man. British Journal of Nutrition, 27:211 (1972).
22. Danforth, E. Dietary Induced Thermogenesis: Control of Energy Expenditure. Life Sciences, 28, 1821-1827 (1981).
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MINERAL ELEMENT ANALYSES OF VARIOUS TROPICAL FORAGES IN¹ GUATEMALA AND THEIR RELATIONSHIP TO SOIL CONCENTRATIONS

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ABSTRACT

A study was conducted to determine the mineral composition of different forage species in three cattle-producing regions of Guatemala and to indicate the relationship found between soil chemistry and the mineral composition of forages. Soil and forage samples were collected at the same site at four, four and six farms within the Northeast, Central and Southwest regions for each season. Sampling periods corresponded to the middle of the rainy season (July-August, 1980) and the middle of the dry season (February-March, 1982). Percentages of mean species nutrient concentrations below critical levels (in parentheses) and suggestive of deficiency for the rainy and dry seasons, respectively, were as follows: Ca (< 0.3%) 14, 69; K (< 0.8%) 0, 8; Mg (< 0.2%) 36, 50; Na (< 0.06%) 36, 77; P (< 0.25%) 50, 54; Fe (< 30 ppm) 0, 0; Co (< 0.1 ppm) 7, 0; Cu (< 10 ppm) 43, 92; Mn (< 40 ppm) 21, 15; Mo (> 6 ppm) 0, 0; Se (< 0.10 ppm) 50, 31; Zn (< 30 ppm) 21, 38; and crude protein (< 7%) 29, 46. Based on analyses, mineral and crude protein concentrations of forage species tended to differ. Soil-forage correlation coefficients of the same mineral not affected by the factor "region" for the rainy season were Na ($r = -0.12$), P ($r = 0.11$) and Cu ($r = -0.05$). In the dry season, the correlations were Na ($r = 0.11$) Mn ($r = -0.04$) and Zn ($r = 0.20$). Results indicate low correlation coefficients between soil and forage minerals, suggesting that soil analyses are not of great value in assessing available mineral supplies.

INTRODUCTION

The mineral composition of forages varies according to factors such as plant age, soil, fertilization practice, species, variety, season and grazing pressure (1). Little relationship has been reported between soil chemistry and mineral composition of native vegetation and

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farm crops (2). Furthermore, mineral interactions between soil, plants and animals in Latin American countries have received little attention. Results from Brazil indicated that correlations between soil and forage minerals were low and, in other cases, nonexistent (3).

The purpose of this investigation was to evaluate the mineral status of cattle-producing regions in Guatemala in relation to composition of different forage species. In addition, the relationship between soil chemistry and the mineral composition of forages in the same regions was studied.

METHODS

Soil and forage samples were collected from different farms within three regions of Guatemala during the rainy and dry seasons. Collections were made at four, four and six farms within the Northeast, Central and Southwest regions, respectively, for each season. The three selected regions are important Zebu-Criollo beef cattle areas with 50% of the total cattle population found in the Southwest region. Sampling periods corresponded to the middle of the rainy season (July-August, 1980) and the middle of the dry season (February-March, 1982). The annual precipitation is extremely varied, ranging from 1,000 to 4,000 mm. A total of 42 soil and 84 forage samples were obtained for each of the sampling periods. Soil and forage samples were collected at the same site. The depth of the soil sample was similar to the length of the majority of the forage root systems (20 cm).

Each of the three composite soil samples for each farm came from 20 to 25 samples. The soil sampling technique used was described by Bahia (4). Although soil samples collected during the two seasons did not come from the exact same spot, they came from the same grazing area of the farm. Soil samples were analyzed according to the procedures used by the University of Florida extension soil testing laboratory in Gainesville, Florida (5). The soil samples were analyzed for organic matter, pH, soluble salts, Al, Ca, K, Mg, Na, P, Fe, Cu, Mn and Zn. Mineral elements were extracted from soils using the Mehlich I extraction solution ($0.05\text{ N HCl} + 0.025\text{ N H}_2\text{SO}_4$), and the soil mineral concentrations, except P, were determined by atomic absorption spectrophotometry (6). Phosphorus was determined by the method of Technicon Industrial Systems (7).

Six composites of the major species of unfertilized forage samples from each farm were collected during each of the sampling periods. Forage samples were processed and analyzed for protein and mineral content according to methods described by Fick et al. (8). Calcium, K, Mg, Na, Fe, Cu, Mn and Zn were analyzed by atomic absorption (6). An atomic absorption spectrophotometer equipped with a graphite furnace (HGA 2100) and D_2 corrector (Perkin-Elmer Model 503) was used to determine forage ^{60}Co and Mo. Phosphorus was determined by the colorimetric method described by Harris and Papat (9) and included by Fick et al (8) as a method for plant P determination. Selenium analyses of forages were carried out using the fluorometric technique described by Whetter and Ullrey (10). Finally, crude protein concentrations in forages were determined by measuring total N, following the method of Technicon Industrial Systems (7).

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The data from each season were analyzed separately and statistically analyzed using a nested design model with proportional subclass numbers (11); however, balanced sampling was obtained. Data were analyzed by the General Linear Model procedures of the Statistical Analysis System (12) in order to detect effects among regions. Nevertheless, the analysis of the forage data did not use species as a variable. Finally, the correlations between soil and forage responses were calculated.

RESULTS AND DISCUSSION

Mineral and crude protein concentrations of forage species collected during the rainy and dry seasons (Tables 1 and 2) tended to differ. However, these differences were not statistically analyzed because the analysis of forage data did not use species as a variable. This was because the distribution of the observations over the fifteen species was quite limited. In addition, due to the limited number of species which occurred in more than one region, there is probably a high correlation between regions and farms within regions with the species effects. Therefore, in this situation, it would be very difficult to obtain meaningful estimates of the effects. Only overall means and standard deviations for each forage species in each season are reported.

Forage crude protein concentrations were generally lower during the dry season. During the rainy season only 29% of forage species were less than the critical concentration of 7%, while 46% of species were less than this value for the dry season. Mean Ca concentrations among forage species ranged from 0.22 to 0.52% during the dry season. However, this range tended to be higher during the dry season. The percentages of mean species below the suggested critical level of 0.3% Ca (13, 14) were 14 and 69 for the rainy and dry season, respectively.

The percentages of species below the K critical concentration ($< 0.8\%$) were low, 8% during the dry season and 0% during the rainy season. Of all species analyzed, only Jaraguagrass (*Hyparrhenia rufa*) was deficient in K during the dry season. Similar results were found by Mtimuni (15) in Malawi, where the only species found to be deficient in K was Jaraguagrass (0.49%).

Of all species analyzed, only five tended to be deficient in Mg ($< 0.2\%$) during the rainy season. However, during the dry season, one-half of the total species were below the critical concentration. Forage species deficient in Na, below the suggested critical level of 0.06% (13) were found during the rainy (36%) and dry seasons (77%). These data are in agreement with a study on the Pacific coast (Retalhuleu) of Guatemala in which 75% of forages were found below the critical concentration of Na and Mg (16).

Kikuyagrass (*Pennisetum clandestinum*) and Elephantgrass were two grasses with high levels of P (0.40 and 0.35%) during the rainy season. The percentages of species below the critical concentration of 0.25% (13, 14) were 50 and 54% for the rainy and dry season, respectively. Mtimuni (15) reported mean P concentration for Jaraguagrass (0.07%) and Pangolagrass (0.23%) in Malawi. These results are in agreement with the present evaluation, in which mean P concentrations

TABLE 1. MACROMINERAL AND CRUDE PROTEIN COMPOSITION OF FORAGE SPECIES COLLECTED IN EACH SEASON

Species ^c	Season	No. of samples	Percent dry matter									
			Ca (< 0.3) ^a		K (< 0.8) ^a		Mg (< 0.2) ^a		Na (< 0.06) ^a		P (< 0.25) ^a	
			Mean ^b	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Cockspur	Rainy Dry	6	0.33 ± 0.15		2.9 ± 0.9		0.29 ± 0.15		0.14 ± 0.08		0.22 ± 0.05	
		2	0.25 ± 0.00		2.3 ± 0.0		0.30 ± 0.00		0.10 ± 0.01		0.32 ± 0.04	
Bermudagrass	Rainy Dry	8	0.35 ± 0.03		2.3 ± 0.7		0.21 ± 0.10		0.08 ± 0.06		0.27 ± 0.09	
		2	0.29 ± 0.05		1.5 ± 0.3		0.12 ± 0.01		0.04 ± 0.01		0.25 ± 0.03	
Bluestem	Rainy Dry	2	0.33 ± 0.01		2.2 ± 0.1		0.11 ± 0.00		0.05 ± 0.04		0.26 ± 0.01	
		14	0.26 ± 0.03		1.0 ± 0.1		0.09 ± 0.03		0.02 ± 0.01		0.09 ± 0.02	
Molassesgrass	Rainy Dry	4	0.47 ± 0.06		2.0 ± 0.3		0.20 ± 0.03		0.06 ± 0.01		0.23 ± 0.04	
		2	0.22 ± 0.01		1.0 ± 0.1		0.18 ± 0.00		0.01 ± 0.00		0.24 ± 0.01	
Bahia grass	Rainy Dry	4	0.52 ± 0.01		1.7 ± 0.4		0.36 ± 0.16		0.05 ± 0.01		0.20 ± 0.03	
		4	0.61 ± 0.15		2.4 ± 0.5		0.36 ± 0.10		0.03 ± 0.03		0.27 ± 0.05	
Signalgrass	Rainy Dry	2	0.34 ± 0.08		3.9 ± 1.4		0.33 ± 0.08		0.18 ± 0.04		0.39 ± 0.04	
		2	0.21 ± 0.02		1.7 ± 0.2		0.23 ± 0.03		0.07 ± 0.04		0.26 ± 0.03	
Stargrass	Rainy Dry	25	0.32 ± 0.12		2.2 ± 1.0		0.16 ± 0.08		0.06 ± 0.04		0.27 ± 0.15	
		22	0.25 ± 0.04		1.3 ± 0.3		0.11 ± 0.03		0.04 ± 0.03		0.20 ± 0.07	
Paspalum	Rainy Dry	4	0.24 ± 0.11		2.2 ± 1.1		0.28 ± 0.12		0.04 ± 0.02		0.33 ± 0.09	
		8	0.38 ± 0.17		2.3 ± 0.4		0.29 ± 0.09		0.03 ± 0.02		0.32 ± 0.03	
Guineagrass	Rainy Dry	4	0.32 ± 0.13		2.1 ± 0.8		0.14 ± 0.04		0.03 ± 0.01		0.24 ± 0.01	
		2	0.33 ± 0.00		0.9 ± 0.1		0.23 ± 0.00		0.04 ± 0.00		0.18 ± 0.02	
Rhodesgrass	Rainy Dry	0										
		2	0.47 ± 0.09		3.5 ± 0.6		0.24 ± 0.04		0.04 ± 0.02		0.47 ± 0.09	
Jaraguagrass	Rainy Dry	6	0.31 ± 0.07		1.1 ± 0.6		0.27 ± 0.09		0.06 ± 0.05		0.14 ± 0.03	
		8	0.28 ± 0.04		0.5 ± 0.1		0.18 ± 0.03		0.02 ± 0.01		0.13 ± 0.04	
Kikuyagrass	Rainy Dry	6	0.50 ± 0.03		2.6 ± 0.7		0.29 ± 0.13		0.09 ± 0.03		0.40 ± 0.02	
		12	0.23 ± 0.03		1.5 ± 0.3		0.18 ± 0.02		0.03 ± 0.02		0.31 ± 0.05	

TABLE 1. - continued.

Species ^c	Season	No. of samples	Percent dry matter									
			Ca (<0.3) ^a		K (<0.8) ^a		Mg (<0.2) ^a		Na (<0.06) ^a		P (<0.25) ^a	
			Mean ^b	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Corn	Rainy	2	0.46 ± 0.07		3.9 ± 0.2		0.20 ± 0.02		0.12 ± 0.01		0.21 ± 0.01	10.3 ± 0.1
	Dry	0										
Elephantgrass	Rainy	1	0.30 ± 0.00		2.5 ± 0.0		0.12 ± 0.00		0.05 ± 0.00		0.35 ± 0.00	7.1 ± 0.0
	Dry	0										
Pangolagrass	Rainy	10	0.22 ± 0.09		1.5 ± 0.9		0.11 ± 0.05		0.22 ± 0.06		0.23 ± 0.10	6.9 ± 1.9
	Dry	4										

^aCritical concentrations (%) according to McDowell et al. (13).^bMean ± standard deviation.

^cCommon names are as follows: Echinochloa polystachia (Cockspur), Cynodon dactylon (Bermudagrass), Andropogon spp. (Bluestem), Melinis minutiflora (Molassesgrass), Paspalum dilatatum (Bahia grass), Brachiaria spp. (Signalgrass), Cynodon plectostachyum (Stargrass), Paspalum spp. (Paspalum), Panicum maximum (Guineagrass), Chloris gayana (Rhodesgrass), Hyparrhenia rufa (Jaraguagrass), Pennisetum clandestinum (Kikyuagrass), Zea mays (Corn), Pennisetum purpureum (Elephantgrass) and Digitaria decumbens (Pangolagrass).

TABLE 2. MICROMINERAL COMPOSITION OF FORAGE SPECIES COLLECTED IN EACH SEASON

Species ^C	Season	No. of samples	ppm, dry matter basis													
			Fe (<30) ^A		Co (<0.1) ^A		Cu (<10) ^A		Mn (<40) ^A		Mo (>6) ^A		Se (<0.1) ^A		Zn (<30) ^A	
			Mean ^B	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Cockspur	Rainy Dry	6	533 ± 439		0.38 ± 0.14		7 ± 3		175 ± 117		2.54 ± 1.85		0.15 ± 0.03		31 ± 13	
		2	1234 ± 121		0.85 ± 0.07		5 ± 1		412 ± 10		2.94 ± 0.83		0.93 ± 0.01		32 ± 3	
Bermudagrass	Rainy Dry	8	633 ± 234		0.47 ± 0.21		12 ± 11		73 ± 33		1.15 ± 0.60		0.25 ± 0.21		36 ± 8	
		2	516 ± 151		0.29 ± 0.03		4 ± 5		52 ± 15		0.71 ± 0.34		0.24 ± 0.12		24 ± 8	
Bluestem	Rainy Dry	2	174 ± 1		0.16 ± 0.05		5 ± 1		37 ± 1		0.42 ± 0.01		0.03 ± 0.01		22 ± 4	
		14	480 ± 209		0.35 ± 0.25		2 ± 1		71 ± 56		0.19 ± 0.04		0.05 ± 0.02		24 ± 14	
Molassesgrass	Rainy Dry	4	673 ± 622		0.41 ± 0.02		19 ± 1		148 ± 116		0.47 ± 0.06		0.05 ± 0.04		40 ± 12	
		2	470 ± 5		0.39 ± 0.01		3 ± 1		31 ± 3		0.31 ± 0.01		1.07 ± 0.02		39 ± 4	
Bahia grass	Rainy Dry	4	165 ± 32		0.35 ± 0.04		7 ± 6		100 ± 22		2.34 ± 0.48		0.31 ± 0.02		35 ± 7	
		4	452 ± 341		0.30 ± 0.11		5 ± 2		126 ± 90		0.55 ± 0.19		0.53 ± 0.48		38 ± 4	
Signalgrass	Rainy Dry	2	311 ± 62		0.38 ± 0.10		23 ± 7		190 ± 51		0.53 ± 0.08		0.18 ± 0.01		64 ± 25	
		2	1765 ± 6		0.92 ± 0.01		6 ± 1		347 ± 28		1.78 ± 0.10		0.94 ± 0.06		49 ± 11	
Stargrass	Rainy Dry	25	308 ± 212		0.38 ± 0.20		10 ± 7		85 ± 30		0.61 ± 0.50		0.10 ± 0.10		31 ± 17	
		22	756 ± 482		0.32 ± 0.16		3 ± 1		81 ± 52		0.39 ± 0.33		0.22 ± 0.38		28 ± 8	
Paspalum	Rainy Dry	4	605 ± 106		0.47 ± 0.12		4 ± 1		76 ± 19		2.73 ± 2.06		0.48 ± 0.56		34 ± 16	
		8	617 ± 405		0.45 ± 0.33		5 ± 3		91 ± 53		1.36 ± 0.86		0.75 ± 0.40		35 ± 6	
Guineagrass	Rainy Dry	4	62 ± 16		0.12 ± 0.05		18 ± 19		23 ± 10		0.96 ± 0.70		0.07 ± 0.05		16 ± 1	
		2	321 ± 26		0.22 ± 0.01		2 ± 1		46 ± 3		0.35 ± 0.02		0.06 ± 0.01		26 ± 3	
Rhodesgrass	Rainy Dry	0														
		2	375 ± 78		0.17 ± 0.02		5 ± 1		39 ± 9		1.61 ± 0.04		0.92 ± 0.01		32 ± 12	
Jaraguagrass	Rainy Dry	6	135 ± 89		0.15 ± 0.08		2 ± 1		86 ± 97		0.51 ± 0.33		0.12 ± 0.05		19 ± 11	
		8	578 ± 246		0.39 ± 0.06		4 ± 2		71 ± 34		0.27 ± 0.09		0.05 ± 0.04		28 ± 5	
Kikuyagrass	Rainy Dry	6	626 ± 598		0.60 ± 0.04		55 ± 61		78 ± 64		0.29 ± 0.02		0.09 ± 0.01		40 ± 20	
		12	550 ± 346		0.39 ± 0.11		37 ± 53		77 ± 54		0.28 ± 0.05		0.81 ± 0.34		56 ± 26	

TABLE 2. - continued.

Species	C	Season	No. of samples	ppm, dry-matter basis													
				Fe (< 30) ^a		Co (< 0.1) ^a		Cu (< 10) ^a		Mn (< 40) ^a		Mo (> 6) ^a		Se (< 0.1) ^a		Zn (< 30) ^a	
				Mean ^b	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Corn		Rainy	2	774 ± 132		0.40 ± 0.08		160 ± 1		107 ± 12		0.16 ± 0.04		0.02 ± 0.00		28 ± 2	
		Dry	0														
Elephantgrass		Rainy	1	36 ± 0		0.07 ± 0		8 ± 0		14 ± 0		0.43 ± 0		0.06 ± 0		20 ± 0	
		Dry	0														
Pangolagrass		Rainy	10	253 ± 216		0.27 ± 0.10		45 ± 66		92 ± 53		0.55 ± 0.42		0.09 ± 0.08		30 ± 11	
		Dry	4	1157 ± 528		0.58 ± 0.18		4 ± 1		154 ± 84		0.38 ± 0.25		0.08 ± 0.06		31 ± 6	

^a Critical concentrations (%) according to McDowell et al. (13) and NRC (14).^b Mean ± standard deviation.

^c Common names are as follows: Echinochloa polystachia (Cockspur), Cynodon dactylon (Bermudagrass), Andropogon spp. (Bluestem), Melinis minutiflora (Molassesgrass), Paspalum dilatatum (Bahia grass), Brachiaria spp. (Signalgrass), Cynodon plectostachyum (Stargrass), Paspalum spp. (Paspalum), Panicum maximum (Guineagrass), Chloris gayana (Rhodesgrass), Hyparrhenia rufa (Jaraguagrass), Pennisetum clandestinum (Kikyuagrass), Zea mays (Corn), Pennisetum purpureum (Elephantgrass) and Digitaria decumbens (Pangolagrass).

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for Jaraguagrass and Pangolagrass during the rainy season were 0.14 and 0.23%, respectively. This suggests the effect of specie on the mineral concentration of forages.

Among the microelements, a high percentage of species were below the critical levels for Cu, Mn, Se and Zn. Mean Cu concentration ranged among forage species from 2 to 160 ppm during the rainy season and from 2 to 37 ppm in the dry season. The percentage of species below the suggested critical concentration of 10 ppm (13, 14) was high for both seasons, with the percentage higher in the dry season (43 vs 92%). It is of importance to note that corn (*Zea mays*) was the specie with the highest amount of Cu and the lowest in Mo during the rainy season. Flores et al. (16) stated that 100% of forages in Retalhuleu, Guatemala were deficient in Cu.

The percentages of mean values for Mn, Se and Zn below the critical levels (in parentheses) suggested by McDowell et al. (13) during the rainy season were as follows (ppm): Mn (< 40) 21; Se (< 0.1) 50; and Zn (< 30) 21. Similarly, during the dry season, these were found to be 15 for Mn, 31 for Se and 38 for Zn.

Gomide (1), in Brazil, indicated differences in mineral composition among forage species. He suggested that it is necessary to make comparisons referring to the species studied in the same experiment to prevent the superimposing of effects of other factors such as soil type, fertilization levels and management. Grazing livestock depend almost entirely upon forages to meet their mineral requirements (13). However, many forage species from this study did not meet mineral requirements for grazing livestock. Therefore, mineral supplementation, apart from common salt, is needed in Guatemala.

Overall mean soil and forage parameters as related to season are presented in Table 3. Fleming (17) listed some of these factors which have an effect on mineral uptake by plants such as acidity, moisture, soil temperature, season, plant species, plant variety and fertilization practices. Correlation coefficients between soil and forage minerals as related to season were calculated but only reported when the factor "region" (Northeast, Central and Southwest regions) had no effect either in soil or in the forage parameters. The correlation coefficients for the same mineral between soil and forage for the rainy season were Na ($r = -0.12$), P ($r = 0.11$) and Cu ($r = -0.05$). Similarly, during the dry season, the following were encountered: Na ($r = 0.11$), Mn ($r = -0.04$) and Zn ($r = 0.20$). These results show low, and in some cases even negative, correlation coefficients between soil and forage minerals. Therefore, soil analyses under these conditions, were of no value in assessing the mineral status of grazing cattle.

These data illustrating low and often negative soil-forage correlations are in agreement with data from Brazil (3). These authors stated correlation coefficients for the same mineral between soil and forage as follows: P ($r = 0.11$), Mg ($r = 0.04$), Fe ($r = 0.12$), Mn ($r = -0.12$) and Zn ($r = 0.30$). This indicated that correlations between soil and plants were low or lacking. McDowell et al. (18) reported soil-forage correlation coefficients in Florida as low or nonexistent as follows:

TABLE 3. OVERALL MEAN SOIL AND FORAGE PARAMETERS AS RELATED TO SEASON

Parameter	Soil				Forage				
	Critical level ^a	Rainy		Dry	Critical level ^a	Rainy		Dry	S.E.
		Mean ^b	S.E.			Mean ^c	S.E.		
Organic matter, %		4.3	1.7	7.7					
pH		6.0	0.4	6.0					
Soluble salts, ppm		738	415	250					
Al, ppm		306	85	342					
Ca, ppm	< 71	2065	464	1654		0.34	0.11	0.28	0.09
K, ppm	< 62	321	127	304		2.2	0.7	1.4	0.4
Mg, ppm	< 30	386	66	388		0.2	0.06	0.17	0.05
Na, ppm		42	11	28		0.09	0.07	0.03	0.02
P, ppm	< 17	13.6	11.0	6.5		0.26	0.09	0.23	0.06
Fe, ppm	< 2.5	41	38	62		378	248	661	262
						0.36	0.16	0.38	0.15
Cu, ppm	< 0.3					21	28	8	15
Mn, ppm	< 5	64	89	54		92	37	93	44
						0.94	0.77	0.59	0.37
						0.14	0.13	0.4	0.04
Zn, ppm	< 1	5.6	3.8	5.9		32	13	33	9
						8.8	2.1	6.4	1.1
						C.P., %			

^a Critical levels (4, 8, 13).
^b Mean and standard error of the mean based on 42 samples for each season (rainy and dry).
^c Mean and standard error of the mean based on 84 samples for each season (rainy and dry).

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Ca ($r = 0.27$), Mg ($r = -0.02$), Na ($r = -0.20$), K ($r = 0.06$), Fe ($r = 0.11$), Mo ($r = 0.14$), Se ($r = -0.14$) and Zn ($r = 0.17$). Similarly, from a detailed geochemical survey in Missouri (19), little relationship was found between soil chemistry and mineral composition of native vegetation and farm crops.

The results lead to the conclusion that the soil mineral system interacts differently than the plant mineral system and that factors affecting mineral uptake by plants, previously described, play an important role in the low correlations between the soil and the plant systems. The general conclusion is that soil mineral analysis cannot be relied on to predict mineral adequacy for grazing livestock.

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REFERENCES

1. Gomide, J.A. Mineral composition of grasses and tropical leguminous forages. In: J.H. Conrad and L.R. McDowell (editors) Latin American Symposium on Mineral Nutrition with Grazing Ruminants. University of Florida, Gainesville, p. 32-40 (1978).
2. Reid, R.L. and Horvath, D.J.. Soil chemistry and mineral problems in farm livestock. A review. *Anim. Feed Sci. Technol.* 5:95-167 (1980).
3. Conrad, J.H., Sousa, J.C., Mendez, M.O., Blue, W.G., and McDowell, L.R. Iron, manganese, sodium and zinc interrelationships in a tropical soil, plant and animal system. In: L.S. Verde and A. Fernández (editors) Fourth World Conference on Animal Production. Buenos Aires, Argentina, p. 38-53 (1980).
4. Bahia, V.G. Techniques of soil sampling and analysis. In: J.H. Conrad and L.R. McDowell (editors) Latin American Symposium on Mineral Nutrition Research with Grazing Ruminants. University of Florida, Gainesville, p. 27-29 (1978).
5. Mitchell, A., Jr. and Rhue, R.D. Procedures used by the University of Florida soil testing and analytical research laboratory. *Soil Sci. Res. Dept.* 79-1:51 (1979).
6. Perkin-Elmer. Analytical methods for atomic absorption spectropho-

NUTRITION REPORTS INTERNATIONAL

- tometry. Perkin-Elmer, Norwalk, CT (1980).
7. Technicon Industrial Systems. Individual/simultaneous determination of crude protein, phosphorus and/or calcium in feeds. Industrial Method No. 605-77A. Tarrytown, New York (1978).
 8. Fick, K.R., McDowell, L.R., Miles, P.H., Wilkinson, N.S., Funk, J.D. and Conrad, J.H. Methods of Mineral Analysis for Plant and Animal Tissues (2nd edition). Department of Animal Science, University of Florida, Gainesville (1979).
 9. Harris, W.D. and Popat, P. Determination of the phosphorus content of lipids. *J. Am. Oil Chem. Soc.* 31:124-127 (1954).
 10. Whetter, P.A. and Ullrey, D.E. Improved fluorometric methods of determining selenium. *J. Assoc. Anal. Chem.* 61:927-930 (1978).
 11. Snedcor, G.W. and Cochran, W.G. Statistical Methods (6th edition). Iowa State University Press, Ames (1973).
 12. SAS Institute, Inc. SAS User's Guide: Basics. Cary, North Carolina (1982).
 13. McDowell, L.R., Conrad, J.H. and Ellis, G.L. Mineral deficiencies and imbalances and their diagnosis. Symposium on Herbivore Nutrition in Sub-Tropics and Tropics - Problems and Prospects. Pretoria, South Africa, p. 67-88 (1984).
 14. NRC. Nutrient Requirements of Domestic Animals No. 4. Nutrient Requirements of Cattle, Sixth Revised Edition. National Academy of Sciences, National Research Council, Washington, D.C. (1984).
 15. Mtimuni, J.P. Identification of mineral deficiencies in soil, plant and animal tissues as constraints to cattle production in Malawi. Ph.D. Dissertation. University of Florida, Gainesville (1982).
 16. Flores, J.R., Flores, J.A., Cabezas, M.T. and Bressani, R. Estado de nutrición mineral del ganado de carne de tres fincas de los departamentos de Retalhuleu y Suchitepequez en la costa del Pacífico de Guatemala. Facultad de Medicina Veterinaria y Zootecnia, Universidad de San Carlos de Guatemala (1978).
 17. Fleming, G.A. 1973. Mineral composition of herbage. In: G.W. Butler and R.W. Bailey (Eds.) Chemistry and Biochemistry of Herbage. Academic Press, New York, p. 529-566 (1973).
 18. McDowell, L.R., Kiatoko, M., Bertrand, J.E., Chapman, H.L., Pate, F.M., Martin, F.G. and Conrad, J.H. Evaluating the nutritional status of beef cattle herds from four soil order regions of Florida. II. Trace minerals. *J. Anim. Sci.* 55:38-47 (1982).
 19. Hemphill, D.D. The Missouri study. In: Geochemistry and the Environment. Vol. II. The Relation of Other Selected Trace

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Elements to Health and Disease. National Academy of Sciences, Washington, D.C., p. 124-131 (1977).

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EFFECT OF FORMALDEHYDE TREATMENT OF DIETARY CASEIN ON SERUM CHOLESTEROL LEVELS IN RATS

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ABSTRACT

Female lean, and male obese Zucker rats were fed high-fat, high-cholesterol and low-fat, cholesterol-free semipurified diets, respectively. The diets contained different nitrogen sources: the rats were fed soy protein, casein, formaldehyde-treated casein or casein plus formaldehyde. Casein caused an increase in serum cholesterol when compared to soy protein. In the male obese Zucker rats fed formaldehyde-treated casein, serum cholesterol levels were lower than in their counterparts fed casein and formaldehyde per se, but the difference did not reach statistical significance at all time points during the experiment. In the female lean Zucker rats, formaldehyde-treated casein caused higher serum cholesterol concentrations than did casein to which formaldehyde had been added. In these female rats the formaldehyde-treatment of casein drastically reduced its apparent digestibility. The apparent digestibilities of native casein and soy protein were not different. We conclude that in rats, the apparent digestibility of proteins is not crucial with respect to their effect on the level of serum cholesterol.

INTRODUCTION

The feeding of semipurified diets containing casein as a protein source produces increased levels of serum cholesterol in rabbits and rats, whereas when soy-protein diets are fed low levels are maintained (1). One hypothesis to explain the differential effect of these proteins purports that their digestibility is crucial (2), but other explanations have also been put forward (3, 4).

Proteins that are not completely digested may interfere with the absorption of bile acids (5), and interrupt the enterohepatic circulation of bile acids, which in turn may result in an enhanced loss of steroids with the feces, and consequently in lower levels of serum cholesterol. This idea would imply that soy protein is less digestible than casein, at least in the distal part of the small intestine where the absorption of bile acids takes place. It has been shown in rabbits that the fecal excretion of steroids is decreased almost immediately (within two days) after soy protein is replaced by casein, and before the concentration of serum cholesterol is increased (6). Furthermore, mice fed soy protein have increased amounts of material in their intestine compared with animals fed casein (7), suggesting a slower rate of digestion of soy protein when compared with casein.

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Formaldehyde treatment of proteins causes cross-linking of the protein chains, and decreases the digestion by pepsin in-vitro (2). Probably such treatment also reduces in-vivo digestibility of proteins in rabbits. In any case, formaldehyde-treatment of casein was found to reduce its hypercholesterolemic effect in rabbits (2). This observation would be in line with the hypothesis described above.

The present study with rats was carried out in an attempt to reproduce the effect of formaldehyde-treated casein observed earlier in rabbits (2). For this purpose we used female lean and male obese Zucker rats. Conditions were chosen so that the differential cholesterolemic effect of casein and soy protein would be maximal. Thus, the lean rats were fed high-fat semipurified diets with added cholesterol (8), whereas the obese rats received low-fat, cholesterol-free diets (9).

MATERIALS AND METHODS

Animals, diets, design of experiment

Female lean and male obese Zucker rats from a colony maintained at the Small Animal Center (CKP) of the Agricultural University were used. The animals were fed a commercial rat diet (RMH-B®, Hope Farms, Woerden, The Netherlands). The rats were kept in groups of 6 animals in cages (60 x 21 x 19 cm) constructed of stainless steel with wire mesh bases in a room with air conditioning (20 °C), controlled lighting (12 hours light/dark cycle) and relative humidity (55 to 65%).

When the animals were aged about 5 weeks, all rats were fed a semipurified diet containing soy protein isolate (Purina protein 500E, Ralston Purina Co., St. Louis, MO) for a period of 29 days until day 0 of the experiment. The female lean Zucker rats were fed the high-fat, high cholesterol diet, and the male obese Zucker rats the low-fat, cholesterol-free diet (Table 1). On day 0 rats were transferred to the diets containing either casein, casein plus formaldehyde or formaldehyde-treated casein; one group of female lean rats remained on the soy-protein diet. The composition of the diets is given in Table 1. The groups were formed so that per sex they had similar distributions of serum cholesterol concentration and body weight on day -2 and day 0, respectively. At day 47 of the experiment the diets were switched as illustrated in Fig. 1, and the animals were kept in groups on these diets for a further 42 days.

On day 90 the female lean Zucker rats were housed individually in metabolism cages for another 15 days, during which, for the last four days, feces and urine were collected.

The diets were offered as meal. Diets and water were fed ad libitum. Feed consumption per group (or per individual animal for the female lean Zucker rats during the last four days of the experiment) and the individual body weights were measured.

Analytical methods

Formaldehyde treatment of casein was performed as described (2). Determination of formaldehyde in the protein preparation and in the diet was carried out as described (2, 10). Pepsin-digestibility of the protein preparations was estimated as described (11). Nitrogen in the protein preparations, feed, feces and urine was measured by the Kjeldahl method (12).

TABLE 1. Composition of the experimental diets (g/100 g of diet)

Ingredient	High-fat, high-cholesterol diets				Low-fat, cholesterol-free diets			
	soy protein	casein	F-casein	casein + F	soy protein	casein	F-casein	casein + F
soy isolate	20.8	-	-	-	20.8	-	-	-
methionine	0.2	-	-	-	0.2	-	-	-
casein	-	21.0	10.5	21.0	-	21.0	10.5	21.0
F-casein	-	-	10.7	-	-	-	10.7	-
formaldehyde	-	-	0.2	0.4	-	-	0.2	0.4
corn starch	41.0	41.0	41.0	41.0	-	-	-	-
sucrose	-	-	-	-	64.0	63.0	62.6	62.6
molasses	-	-	-	-	5.0	5.0	5.0	5.0
soybean oil	-	1.1	1.1	1.1	-	1.1	1.1	1.1
coconut fat	9.3	9.0	9.0	9.0	-	-	-	-
cholesterol	1.2	1.2	1.2	1.2	0.3	-	-	-
sawdust	19.5	18.5	18.1	18.1	1.7	1.7	1.7	1.7
sodium chloride	0.6	0.8	0.8	0.8	0.6	0.8	0.8	0.8
constant components*	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4

The high-fat, high-cholesterol diets were fed to the female lean Zucker rats; the low-fat, cholesterol-free diets were fed to the male obese Zucker rats.

*The constant components consisted of (g/100 g of diet), dicalcium phosphate, 2.9; magnesium carbonate, 0.3; magnesium oxide, 0.2; potassium bicarbonate, 1.8; vitamin premix, 1.2; mineral premix, 1.0. The diets were formulated in such a manner to take into account the analysed amounts of fat and sodium in the soy protein and casein preparations. The compositions of the vitamin and mineral premixes have been described elsewhere (2).

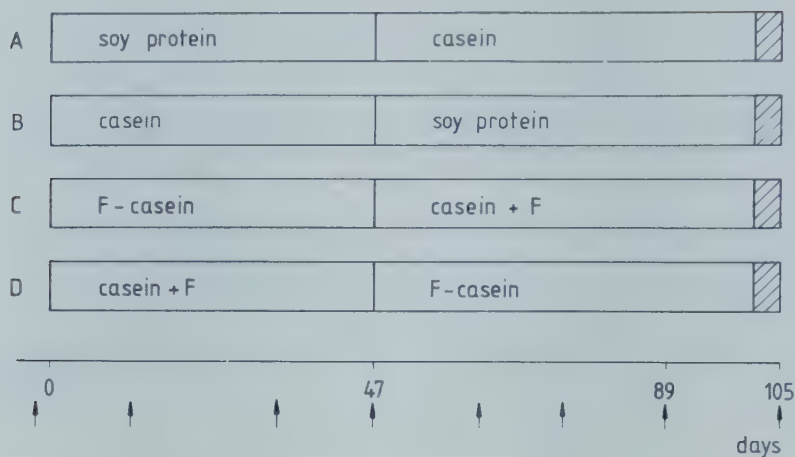


Fig. 1. Design of the experiment. On day 0, when they were aged about 9 weeks, all animals had been fed a semipurified diet containing soy protein for 29 days. The female lean Zucker rats received semipurified diets with a high-fat, high-cholesterol background, whereas the diets of the male obese Zucker rats were essentially cholesterol-free and low in fat. Arrows indicate the days on which blood was sampled. During the last four days of the experimental period (days 101 to 105; hatched part of the bar) feces and urine of the lean Zucker rats were collected for analysis of N and dry matter. The lean rats were used in all dietary groups; the obese rats were used in the F, C and D part of the experiment. F = formaldehyde; F-casein = formaldehyde-treated casein.

The apparent digestibility (in percent) of dry matter and nitrogen was calculated as the amount digested (ingested minus excreted in the feces) divided by the amount ingested times 100. Nitrogen retention (in percent) was calculated as the amount of nitrogen ingested minus nitrogen excreted in feces and urine divided by the amount ingested times 100.

Blood samples were taken in the non-fasting state by orbital puncture under light diethyl-ether anesthesia between 08.00 and 10.00 hours on the days indicated in Fig. 1. Serum cholesterol was measured enzymatically, using the kit (Monotest) purchased from Boehringer-Mannheim GmbH, FRG.

RESULTS

Table 2 shows that formaldehyde-treatment of casein drastically reduced the amount of pepsin-digestible protein. The amount of bound formaldehyde was similar to that in a previous study (2), but in the present study pepsin-digestibility was much more reduced. The reason for this discrepancy is not clear.

The formaldehyde content of the diets was measured. The diets to which no formaldehyde was added did not contain detectable amounts of formaldehyde. In the high-fat diets containing formaldehyde-treated casein and casein plus added formaldehyde 0.29 and 0.14% (by weight) of formaldehyde were found, respectively. In the low-fat diets containing the treated casein and casein plus formaldehyde the values were 0.25 and 0.10%, respectively. It is likely that part of the free formaldehyde evaporated during preparation and storage of the diets.

TABLE 2. Characteristics of casein and the formaldehyde-treated casein

	(g/100 g)	
	Casein	F-casein
Total formaldehyde	-	1.9
Bound formaldehyde	-	1.3
Crude protein	86.4	80.4
Pepsin-digestible protein	84.4	2.1

F-casein = formaldehyde-treated casein.

Acid-casein was used (DMV B.V., Veghel, The Netherlands).

During days 0 to 12 of the experiment the rats in the groups receiving the diets containing formaldehyde-treated casein had this preparation as the only source of protein. The body weight of the animals fell markedly (by 23 ± 1 g in the female lean Zucker rats; $n = 6$, and by 33 ± 2 g in the obese rats; $n = 5$, means \pm SE). We then decided to feed the diets containing the treated casein and the diet with added formaldehyde in a 1:1 ratio for the rest of the experimental period.

At day 46 the rats fed formaldehyde-treated casein had a significantly lower body weight than those fed casein plus formaldehyde, but this difference was not seen at day 88 of the experiment (Table 3). This time course was essentially similar in the lean females and obese males. Body-weight gain was significantly higher in the groups fed casein plus formaldehyde than in those fed formaldehyde-treated casein.

In the female lean Zucker rats casein produced somewhat higher rates of body-weight gain than soy protein (Table 3). This has also been found earlier (9).

When compared with soy protein, casein consistently caused elevated levels of serum total cholesterol in the female lean Zucker rats (Table 4). In the male obese Zucker rats time effects cannot be excluded, but there was a slight decrease in serum cholesterol after transferring the animals from the casein diet to the soy-protein diet on day 47.

When considering the effect of formaldehyde-treatment of casein on serum cholesterol, it should be realized that the rats had been fed the treated protein as a sole source of protein up until day 12 of the experiment. Up until that day growth performance of the rats was not good; the animals even lost weight. Thus effects on serum cholesterol may be biased. It is reasonable to propose that after day 47, when body weights of rats fed either formaldehyde-treated casein or casein plus formaldehyde were not very different (Table 3), the serum cholesterol values show a true effect of formaldehyde-treatment of dietary casein. Table 4 illustrates that in the female lean Zucker rats treatment of casein with formaldehyde resulted in higher serum cholesterol concentrations (except on day 74) than did the addition of pure formaldehyde to the diet. This is also illustrated in Fig. 2. In the male obese Zucker rats fed treated casein serum cholesterol levels were lower than in those fed formaldehyde per se, but the difference only reached statistical significance at days 62 and 74.

TABLE 3. Growth and feed intake of rats fed the experimental diets

	Body weight (g)			Weight gain (g/day)		Feed intake (g/day)	
	day 0	day 46	day 88	days 0 to 46	days 46 to 88	days 0 to 46	days 46 to 88
Female lean Zucker rats							
A, Soy-Casein group (6)	126± 2	168± 2	188± 3	0.93±0.02	0.47±0.03	14.1	13.3
B, Casein-Soy group (5)	126± 2	172± 2	190± 2	1.00±0.07	0.41±0.06	13.6	14.0
C, F-Casein - Casein + F group (6)							
D, Casein + F - F-Casein group (6)	125± 2	164± 3 ^b	188± 4	0.83±0.02 ^b	0.58±0.04	13.7	13.6
	126± 2	176± 3	190± 4	1.07±0.04	0.35±0.02 ^b	13.8	14.7
Male obese Zucker rats							
B, Casein-Soy group (5)	259±24	477±25	545±23	4.74±0.12	1.64±0.29	27.8	30.9
C, F-Casein - Casein + F group (5)							
D, Casein + F - F-Casein group (5)	276±21	403±16 ^b	502±19	2.77±0.18 ^b	2.34±0.13	23.4	30.0
	267±20	476±10	545±13	4.53±0.37	1.65±0.11 ^b	26.8	32.0

Results are expressed as means ± SE; the number of animals per group is indicated in parentheses. For experimental design, see Fig. 1.
^aSignificantly different from the group fed soy protein; ^bsignificantly different from the group fed casein + F (P<0.05; two-tailed Student's t test).

TABLE 4. Serum cholesterol concentrations in rats fed the experimental diets

	Serum total cholesterol (mmol/l)									
	day -2	day 12	day 33	day 47	day 62	day 74	day 89	day 105		
Female lean Zucker rats										
A, Soy-Casein group	3.08±0.12	2.72±0.08	2.78±0.06	2.66±0.09	3.53±0.16 ^a	3.54±0.17 ^a	3.72±0.09 ^a	3.92±0.12 ^a		
B, Casein-Soy group	3.06±0.13	3.29±0.18 ^a	3.75±0.19 ^a	3.31±0.17 ^a	2.88±0.22	2.73±0.17	2.97±0.12	3.19±0.19		
C, F-Casein - Casein + F group	3.07±0.11	3.19±0.19	5.19±0.41	4.59±0.49	3.80±0.22	4.53±0.37	3.64±0.16	3.80±0.19		
D, Casein + F - F-Casein group	3.01±0.12	3.65±0.06	3.98±0.19	4.22±0.22	4.28±0.29	4.36±0.25	4.67±0.59 ^b	5.43±0.96 ^b		
Male obese Zucker rats										
B, Casein-Soy group	3.34±0.19	3.36±0.24	5.15±0.41	4.82±0.36	4.28±0.22	4.57±0.30	4.47±0.33	4.78±0.35		
C, F-Casein - Casein + F group	3.34±0.20	2.19±0.14	5.25±0.41	5.12±0.49	5.51±0.23	6.34±0.34	5.69±0.63	8.56±2.23		
D, Casein + F - F-Casein group	3.30±0.18	4.21±0.24	4.99±0.26	5.14±0.20	4.71±0.24 ^b	4.93±0.13 ^b	5.31±0.20	6.15±1.20		

Results are expressed as means ± SE; the number of animals per group is indicated in parentheses. For experimental design, see Fig. 1. ^aSignificantly different from the group fed soy protein; ^bsignificantly different (calculated only for days 62 to 105) from the group fed casein + F ($P < 0.05$; two-tailed Student's t test).

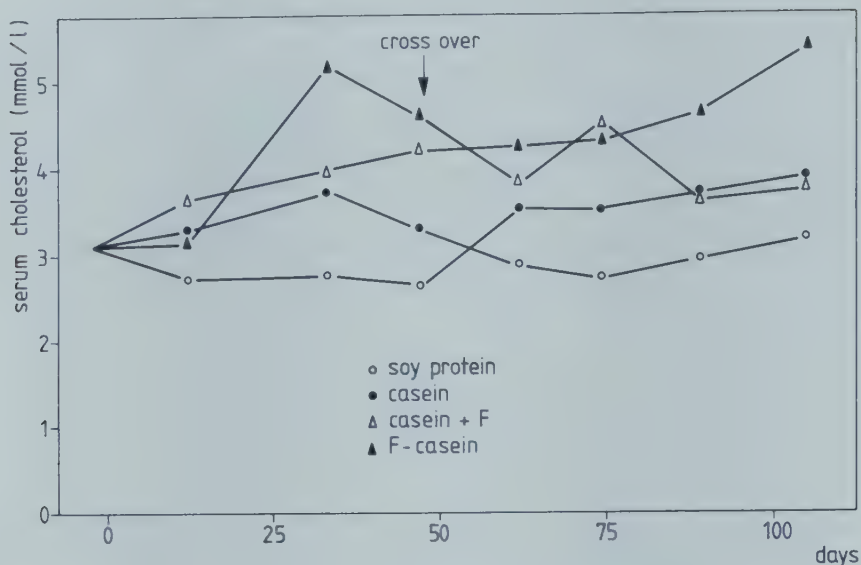


Fig. 2. Time course of the average concentration of serum cholesterol in the female lean Zucker rats fed the high-fat, high-cholesterol diets. ○, group fed soy protein; ●, group fed casein; △, group fed casein plus formaldehyde; ▲, group fed formaldehyde-treated casein. Data are taken from Table 4.

Table 5 shows the results of the digestibility experiment with the female lean Zucker rats. Feed intake of the animals fed casein and soy protein were identical. The rats fed casein had a somewhat lower excretion of nitrogen with the feces than the rats fed soy protein, but they tended to excrete more nitrogen with the urine. As a result, nitrogen retention was lower in the rats fed casein. Digestibility of dry matter and nitrogen were similar on diets containing casein or soy protein.

The data from the digestibility experiment in Table 5 show that the female lean Zucker rats fed formaldehyde-treated casein consumed 36% more feed than the rats fed the diet containing casein plus formaldehyde. This difference in feed intake could theoretically explain in itself the higher serum cholesterol levels in the rats fed casein treated with formaldehyde (Table 4, Fig. 2), because the rats also ingested more cholesterol. During days 46 to 88 of the experiment the effect of formaldehyde-treated casein on feed intake was much smaller (Table 3), and thus it is unlikely that the increased serum cholesterol levels were the result of the increased intake of feed.

When compared to the diet containing casein and formaldehyde, the diet containing casein treated with formaldehyde resulted in a drastically increased output of nitrogen in the feces (Table 5). This effect cannot be explained by the higher feed intake, as the apparent digestibility of nitrogen was significantly lower in rats fed formaldehyde-treated casein. Formaldehyde-treatment of casein caused a significantly lower loss of nitrogen with the urine than the addition of formaldehyde per se to the diet containing casein; this effect resulted in a higher retention of nitrogen. It would appear that rats regulate degradation of proteins in the body, and thus nitrogen output with the urine, on the basis of the availability of protein in the diet.

TABLE 5. Results of the digestibility experiment (days 101 to 105; Fig. 1) performed with the female lean Zucker rats

	Dietary protein			
	Soy protein (n=5)	Casein (n=6)	Casein + F (n=6)	F-Casein (n=6)
Serum cholesterol ¹ (mmol/l)	3.19± 0.42	3.92± 0.30 ^a	3.80± 0.47	5.43± 2.35 ^b
Body weight change (g/day)	+0.40± 1.10	-0.25± 1.04	+0.08± 0.90	+1.75± 1.39
Feed intake				
total feed (g/day)	11 ± 1	11 ± 1	11 ± 1	15 ± 2 ^b
dry matter (g/day)	10 ± 1	10 ± 1	10 ± 1	14 ± 2 ^b
nitrogen (mg/day)	330 ± 30	330 ± 30	330 ± 30	450 ± 60 ^b
Fecal output				
total output (g/day)	5.9 ± 0.5	5.2 ± 0.3	5.0 ± 0.4	8.2 ± 1.2 ^b
dry matter (g/day)	3.0 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	4.4 ± 0.5 ^b
nitrogen (mg/day)	41 ± 2	36 ± 2 ^a	43 ± 8	212 ± 24 ^b
Apparent digestibility				
dry matter (% of intake)	71 ± 1	72 ± 1	73 ± 1	68 ± 1 ^b
nitrogen (% of intake)	88 ± 1	89 ± 1	87 ± 2	54 ± 1 ^b
Urinary output				
total output (g/day)	4.8 ± 1.2	5.6 ± 0.9	5.7 ± 0.6	4.9 ± 1.6
dry matter (g/day)	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
nitrogen (mg/day)	208 ± 42	250 ± 17	239 ± 14	122 ± 18 ^b
Nitrogen retention (% of intake)	27 ± 8	15 ± 6 ^a	17 ± 3	27 ± 2 ^b

Results are expressed as means ± SD.
¹day 105. ^aSignificantly different from the group fed soy protein; ^bsignificantly different from the group fed casein + F (P<0.05; Student's *t* test).

DISCUSSION

It is clear that the hypocholesterolemic effect of formaldehyde-treatment of casein found earlier in rabbits (2) did not occur in rats under the conditions described. As to the reasons for this discrepancy we can only speculate. It is possible that the effect of formaldehyde treatment of casein, that is cross-linking of the protein chains of casein, is species-dependent. This may then imply that the mechanisms underlying the different cholesterolemic effects of soy protein and casein also differ between rabbits and rats.

With regard to a possible difference of the response to casein between rabbits and rats, another hypothesis may be of relevance. The hypercholesterolemic effect of casein may be related to its phosphorylation state (4). It is proposed that in the small intestinal lumen phosphopeptides derived from casein compete with bile acids and biliary micelles for binding to insoluble calcium phosphate. This dietary-casein-induced competition increases the availability of steroids for reabsorption, which eventually results in an increase in serum cholesterol concentration. As predicted by this hypothesis casein-induced hypercholesterolemia in rabbits has been found to be calcium dependent (13). Moreover, in-vitro binding studies using casein and dephosphorylated casein have shown that this proposed mechanism is indeed dependent on the phosphorylation state of casein (4). Since intestinal alkaline phosphatase is able to dephosphorylate casein (Van der Meer, unpublished), it has been suggested (4) that this enzyme could be involved in a species dependence of the response of serum cholesterol to casein. Since rabbits have a low intestinal alkaline phosphatase activity (14), cross-linking of casein may only affect its proteolysis and consequently binding of casein-derived phosphopeptides to insoluble calcium phosphate. However, in rats the situation may be different as these animals have a high activity of alkaline phosphatase (14). In rats cross-linking of casein may not only inhibit its proteolysis but also its dephosphorylation by intestinal alkaline phosphatase. Thus in rats cross-linking could have two opposite effects on the hypercholesterolemic potential of casein. The hypercholesterolemic effect of formaldehyde-treatment of casein observed in the present study indicates that inhibition of dephosphorylation of casein is the major effect of cross-linking the dietary protein.

In sum, the idea that protein digestibility per se would be involved in determining the cholesterolemic effects of dietary proteins, is not supported by the present study. Formaldehyde-treatment of casein drastically reduced the in-vivo apparent digestibility of the protein in the female lean Zucker rats, but serum cholesterol levels were not decreased when compared with animals fed casein and formaldehyde per se.

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REFERENCES

1. West, C.E., Beynen, A.C., Terpstra, A.H.M., Scholz, K.E., Carroll, K.K. and Woodward, C.J.H. The nature of dietary protein and serum cholesterol. *Atherosclerosis* 46, 253 (1983).
2. West, C.E., Beynen, A.C., Scholz, K.E., Terpstra, A.H.M., Schutte, J.B.,

- Deuring, K. and Van Gils, L.G.M. Treatment of dietary casein with formaldehyde reduces its hypercholesterolemic effect in rabbits. *J. Nutr.* 114, 17 (1984).
3. Kritchevsky, D., Tepper, S.A., Czarnecki, S.K. and Klurfeld, D.M. Atherogenicity of animal and vegetable protein - Influence of the lysine to arginine ratio. *Atherosclerosis* 41, 429 (1982).
4. Van der Meer, R. Is the hypercholesterolemic effect of dietary casein related to its phosphorylation state? *Atherosclerosis* 49, 339 (1983).
5. Sklan, D., Budowski, P. and Hurwitz, S. Absorption of oleic and taurocholic acids from the intestine of the chick. Interactions and interference by proteins. *Biochim. Biophys. Acta* 573, 31 (1979).
6. Beynen, A.C., Winnubst, E.N.W. and West, C.E. The effect of replacement of dietary soybean protein by casein on the fecal excretion of neutral steroids in rabbits. *Z. Tierphysiol., Tierernährg. u. Futtermittelkde* 49, 43 (1983).
7. Roy, D.M. and Schneeman, B.O. Effects of soy protein, casein and trypsin inhibitor on cholesterol, bile acids and pancreatic enzymes in mice. *J. Nutr.* 111, 878 (1981).
8. Terpstra, A.H.M., Van Tintelen, G. and West, C.E. The effect of semi-purified diets containing different proportions of either casein or soybean protein on the concentration of cholesterol in whole serum, serum lipoproteins and liver in male and female rats. *Atherosclerosis* 42, 85 (1982).
9. Beynen, A.C., Terpstra, A.H.M., West, C.E. and Van Tintelen, G. The concentration of serum cholesterol in rats fed cholesterol-free, low-fat semipurified diets containing either casein or soybean protein. *Nutr. Rep. Int.* 28, 363 (1983).
10. Bremanis, E. Die photometrische Bestimmung des Formaldehyds mit Chromotropsäure. *Z. Anal. Chem.* 130, 44 (1949).
11. Anonymous. Determination of crude protein soluble in pepsin under acidic conditions. *Off. J. Eur. Communities No. L 123*, 11 (1972).
12. Henry, R.J., Cannon, D.C. and Winkelman, J.W. *Clinical Chemistry: Principles and Technics*. Harper & Row, New York 1974 p. 409.
13. Van der Meer, R., De Vries, H., West, C.E. and De Waard, H. Casein-induced hypercholesterolaemia in rabbits is calcium-dependent. *Atherosclerosis* in press (1985).
14. Mc Comb, R.B., Bowes, Jr. G.N. and Posen, S. *Alkaline Phosphatase*. Plenum Press, New York 1979 p. 81.

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ACUTE EFFECTS OF NALTREXONE ON ENERGY BALANCE AND THERMOREGULATION IN RATS.

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ABSTRACT

The effect of acute naltrexone administration on thermoregulatory function was examined in male rats. In a crossover design, Sprague-Dawley rats were injected (ip) with either saline or 10 mg/kg naltrexone and rectal and tail temperatures monitored every 10 minutes for a 2-hr period. In addition, the effects of the acute administration of naltrexone on O_2 consumption, CO_2 production, thermal conductance (C) and the thermal circulation index (i) were determined. Following naltrexone administration, rectal temperatures decreased, reaching their lowest point 30 min post-injection, returning to saline levels by 100 min post-injection. There were no significant modifications in tail temperature following naltrexone injections. Naltrexone led to a significant suppression in both O_2 consumption and CO_2 production. As there were proportional decreases in both O_2 and CO_2 there were no significant modifications in RQ. C was not modified as a function of naltrexone injections. In contrast, animals exhibited a sharp rise in i after naltrexone administration, which peaked 20 min post-injection, and returned to saline levels by 80 min after drug administration. As naltrexone has its effects by blocking the endogenous opioid system, these data suggest a role for the opioid peptides in short-term regulation of energy metabolism and thermoregulation.

INTRODUCTION

Both central and peripheral infusions of opioid agonists result in dose-related alterations in thermoregulatory function. These drugs are reported to produce hypothermia, hyperthermia or a biphasic response in temperature depending on the dose of drug administered, the species of animals tested, the ambient temperature and the route of administration (1, 2). In general, in rats, low doses of opioids result in hyperthermia, while higher doses lead to hypothermia or biphasic changes in temperature (3, 4).

There is some disagreement over whether or not the effect of the opioids on temperature regulation can be blocked by the

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administration of opioid antagonists. While it appears that the hypothermic effects of the opioids can be reversed by naloxone administration (5, 6), the effects of naloxone on opioid-induced hyperthermia are more controversial (7, 8). In addition to examining the effects of opioid antagonists on opioid-induced changes in thermoregulatory function, recent studies have revealed that both naloxone and naltrexone alone, can produce hypothermia in rats. However, the doses of opioid antagonists employed for these effects were significantly higher than those needed to block opioid-induced hyperthermia (2, 9).

In most of the studies described above, investigators have monitored rectal temperature following acute drug administration. To our knowledge no thermoregulatory mechanisms such as 'core-shell' temperature relationships, changes in thermal conductance or examination of the 'vessel' component of thermoregulation have been studied following acute opioid antagonist administration. Moreover, the only report of changes in heat production observed following the acute injection of these drugs was noted as an unpublished result in a paper by Mandenoff et al. (10). These investigators reported that at thermoneutrality there were no effects of acute naloxone administration on resting oxygen consumption in rats.

The purpose of the present study was to examine the acute effects of naltrexone on overall thermoregulatory function in rats. Rectal temperature was simultaneously monitored with tail temperature to allow a more systematic examination of the 'core-shell' relationship following naltrexone injections. In addition, the effects of naltrexone on heat production were determined by indirect calorimetry with measurements of O_2 consumption, CO_2 production taken and respiratory quotient (RQ) determined.

MATERIALS AND METHODS

Ten male Sprague-Dawley rats (CD outbred, Charles River Breeding Laboratories, Wilmington, MA), weighing an average of 330 g at the beginning of the study, were used. Animals were housed individually in standard laboratory cages in a temperature-controlled room (23-25°C) maintained on a 12:12 hour light-dark cycle (lights on 0800 h).

Animals were divided into two groups matched on the basis of body weight. The Sucrose + Chow-fed group (n=5) was given ad lib access to ground Purina Rodent Chow no. 5001 (caloric density=3.6 kcal/g), water and a 32% sucrose solution (1.28 kcal/ml). The sucrose solution was prepared from commercial-grade sugar and tap water on a weight per volume basis. The Chow-fed group (n=5) received only Purina Chow and water. The Sucrose + Chow-fed group was included in the present study, as previous work suggested that opioid antagonists may have more profound effects on hyperphagic than on normophagic animals (11, 12). Animals given access to sucrose in

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addition to the standard laboratory diet typically consume 10-20 % more calories per day than animals with access only to chow (13).

Animals were maintained on their respective diets for a 3-month period prior to testing for the effects of acute naltrexone injections on thermoregulatory function. Animals were tested in a crossover design. On the first test day, half the animals in each dietary group were given intraperitoneal (ip) injections of naltrexone hydrochloride (Endo Laboratories, Garden City, NY). Naltrexone was injected in a dose (10 mg/kg) which in preliminary studies was found to modify thermoregulatory function. Naltrexone was dissolved in nonpyrogenic saline to a concentration that allowed studied doses to be administered in a volume of 0.1ml/100g body weight. The remaining animals were given ip injections of an equal volume of saline. Core (rectal) and shell (tail) temperatures were monitored both prior to injections, and every ten minutes during the two-hour period following injections. One week later, the same procedure was repeated, however animals that had previously received saline were now injected with naltrexone, while those that had been injected with naltrexone, now received saline. In addition, gas exchange (O_2 consumption and CO_2 production) measurements were taken both prior to and following the same dose of naltrexone (10 mg/kg).

Body temperatures were monitored on a Tele-thermometer (model 73 TA, Yellow Springs Instr. Co., Yellow Springs, OH). Rectal temperatures were taken with a thermistor probe (#701) inserted 6 cm into the rectum. A small surface, teflon-covered stainless steel disc temperature probe (#727) was used to monitor tail temperatures.

Gas exchange was performed in an open-circuit system. Animals were placed in metabolic chambers for 30 minutes for baseline measurements. Following the baseline period, animals were injected with naltrexone and placed in the chambers for another 30-minute period to determine the effects of the drug on gas exchange. The air in the chambers was maintained at 23-25°C, equal to the air temperature in the animal room. The metabolic chamber (glass dessicator) was equipped with inlet and outlet ports 1/4 inch in diameter. The expired air from the animals was mixed with room air flowing through the metabolic chamber. This mixture of gases was drawn from the chamber by a glass sampling pump (AS-300, Spectrex) equipped with rheostatic rough and fine adjusters for maintaining constant air flow. Air was then passed successively through a drying cylinder (4 mesh anhydrous $CaSO_4$, drierite, Hammond), a 7 micron brass filter and a fine metering valve (Nupro Co.) to a paramagnetic oxygen analyzer (Beckman, model 755) and to a non-dispersive Infrared CO_2 Analyzer (Beckman, model 864) at flow rates of 800 ml/min for each analysis. The flow rate was constantly monitored by an electronic mass flowmeter with a digital scale (Matheson, model 8160). The output of both the O_2 analyzer and CO_2 analyzer were recorded on a dual chart recorder (Beckman, model 8720A) as percent gas concentrations with a chart speed of 38 mm/min.

The gas analyzers were calibrated with room air (20.93% O_2 and

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0.03% CO₂) and span gas (16% O₂ and 2% CO₂, Matheson). Each animal was placed in the chamber for a 30-minute acclimation period followed by a 30-minute continuous monitoring test period. All data were converted to standard temperature and pressure, dry.

Thermal conductance and thermal circulation index. Thermal conductance (C) expressed in units of weight-specific oxygen uptake (14) and the thermal circulation index (i), indicating the 'vessel' component of thermoregulation (15) were calculated for each animal from the experimental data by the following formulas:

$$C = M/T_{\text{rect}} - T_{\text{air}}$$

where M is heat flow expressed as ml O₂/g/hr,
T_{rect} = rectal temperature, and T_{air} = air temperature,

$$i = T_{\text{tail}} - T_{\text{air}}/T_{\text{rect}} - T_{\text{tail}}$$

where T_{tail} is tail temperature, T_{rect} is rectal temperature and T_{air} is air temperature.

Statistical Analysis. The differences between Sucrose + chow-fed and Chow-fed groups were determined with analysis of variance followed by a posteriori multiple comparison tests. The remaining differences were determined using a t test for paired data. Differences were considered significant when p was less than 0.05.

RESULTS

Body Weight. Animals given access to sucrose + chow had significantly higher body weights than Chow-fed animals. At the time of testing, Sucrose + chow-fed animals weighed an average of 613.5±25.3g, while Chow-fed animals weighed an average of 553.0±23.7g.

Effects of naltrexone on body temperature. As there were no significant differences between Sucrose + chow-fed and Chow-fed animals on either baseline or post-injection rectal or tail temperatures, data from the two groups were combined. Prior to injections, rectal temperatures were stable at 38.6°C. Saline injections had no effect on rectal temperatures over the 2-hour test period. In contrast, naltrexone produced an immediate decrease in rectal temperature. Rectal temperatures reached their lowest point (37.6°C) at 30 min post-injection, gradually returning to baseline levels by 100 min after administration of the drug (Fig. 1).

Animals displayed a similar response in tail temperature following the administration of both saline and naltrexone. Following injections, animals exhibited an initial increase in tail temperature which peaked 20 to 40 min post-injection. This initial rise was followed by a decline in tail temperature to pre-injection

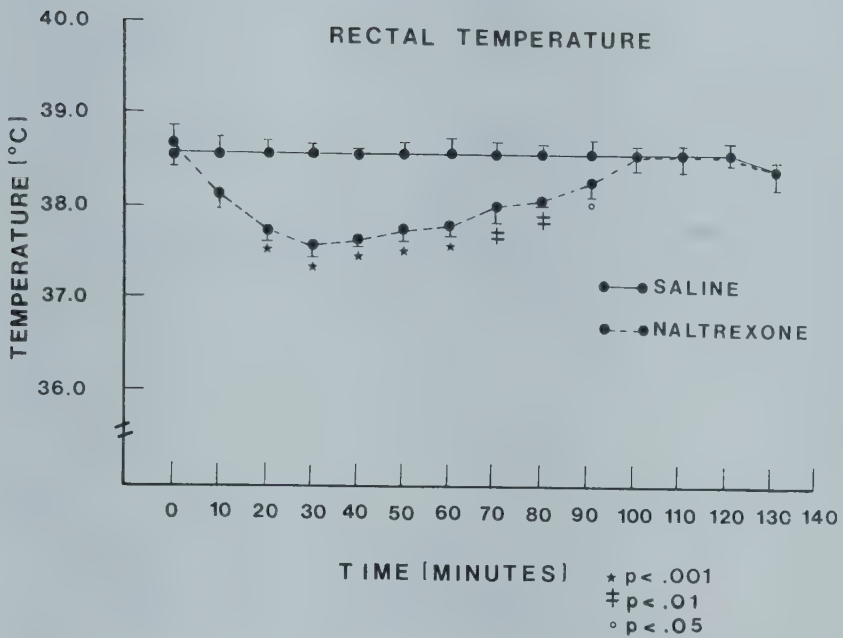


Figure 1. Mean rectal temperatures \pm S.E.M. monitored every 10 minutes over a 2-hour period of rats injected with either saline or 10 mg/kg naltrexone.

levels by 90 to 100 min post-injection. While not significant, due to the great amount of variability, this response appeared more pronounced following naltrexone administration, than following saline injections (Fig.2).

The effects of naltrexone on O_2 consumption, CO_2 production and RQ. As with rectal and tail temperatures, no statistically significant differences were observed in gas exchange measurements between Sucrose + chow-fed and Chow-fed animals. Acute naltrexone injections significantly decreased O_2 consumption and CO_2 production in both Sucrose + Chow-fed and Chow-fed animals. As there were proportional decreases in both O_2 consumption and CO_2 production, there were no significant modifications in RQ (Fig. 3).

Effects of naltrexone on thermal conductance (C) and the thermal circulation index (i). In the present study, thermal conductance did not vary as a function of drug administration (Table 1). Changes in the thermal circulation index are presented in figure 4. It can be seen that i was equal prior to both saline and naltrexone injections. However, when animals received naltrexone, they exhibited a sharp rise in i which peaked 20 min following injections. Over the next 60 min period, i gradually returned to pre-injection levels. While animals showed slight changes in i following saline injections, this increase was significantly less than after naltrexone administration. Following saline injections,

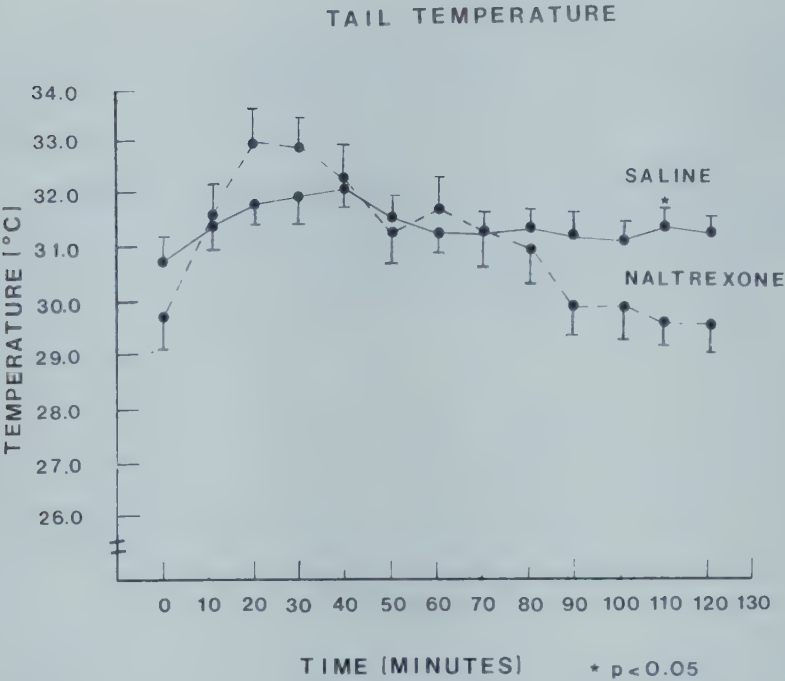


Figure 2. Mean tail temperatures \pm S.E.M. monitored every 10 minutes over a 2-hour period of rats injected with either saline or 10 mg/kg naltrexone.

animals displayed a peak i within 40 min, which returned to pre-injection levels 10 min later (Fig. 4).

DISCUSSION

The present study confirmed previous observations that acute peripheral administration of naltrexone produces hypothermia in rats maintained at normal environmental temperatures (23-25°C) (1). Changes observed in rectal temperature following naltrexone administration occurred in two stages, an initial decrease in rectal temperature, with temperatures dropping to their lowest point by 30 min post-injection, followed by a recovery period, with rectal temperatures returning to pre-injection levels within the next 60 min. As rectal temperatures dropped following naltrexone injections, tail temperatures increased, reaching their highest point 20 to 30 min after administration of the drug. As a similar, but lesser increase in tail temperature was observed following saline injections, it is possible that the intraperitoneal injection itself leads to a reflexive rise in tail temperature. However, the effects of naltrexone were greater in magnitude and longer lasting than those

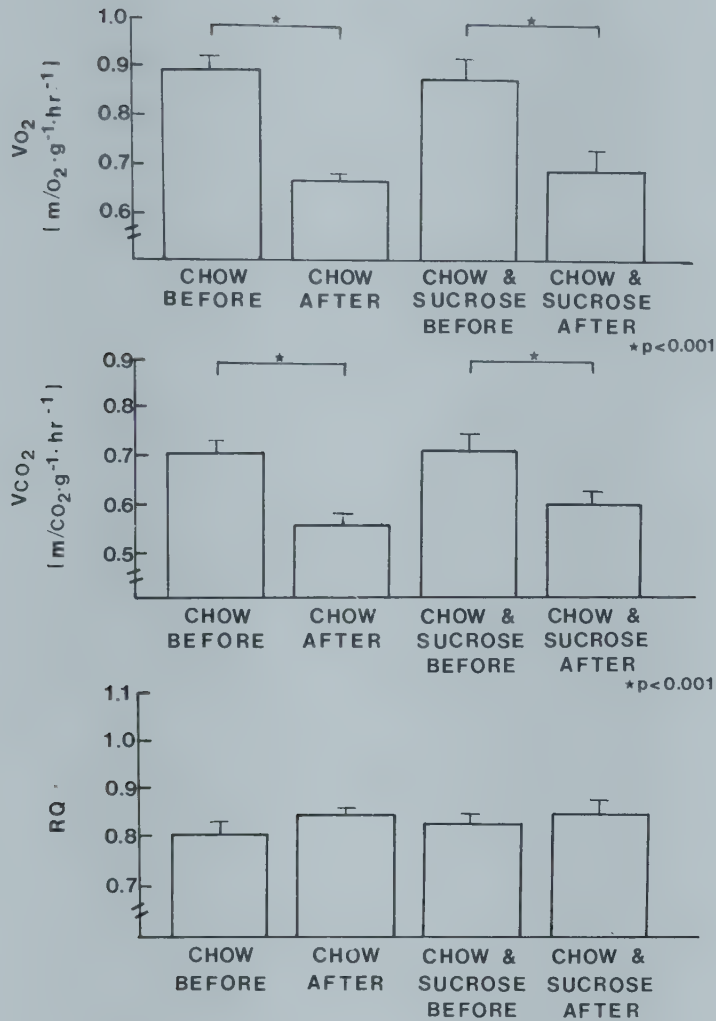


Figure 3. Mean oxygen consumption \pm S.E.M (top), carbon dioxide production \pm S.E.M (middle) and RQ \pm S.E.M (bottom) of Chow-fed and Sucrose + Chow-fed animals, for a 30-minute prior to and a 30-minute period after an injection of 10 mg/kg naltrexone. * $p < 0.001$.

Table I. MEAN THERMAL CONDUCTANCE (C) \pm S.E.M (ml O_2 /g/hr/ $^{\circ}$ C) PRIOR TO (BASELINE PERIOD) AND AFTER (DRUG PERIOD) EITHER AN INJECTION OF SALINE OR 10 MG/KG NALTREXONE.

	BASELINE PERIOD	DRUG PERIOD
Saline-injected	0.0610 \pm 0.0016	0.0613 \pm 0.0016
Naltrexone-injected	0.0634 \pm 0.0020	0.0567 \pm 0.0038

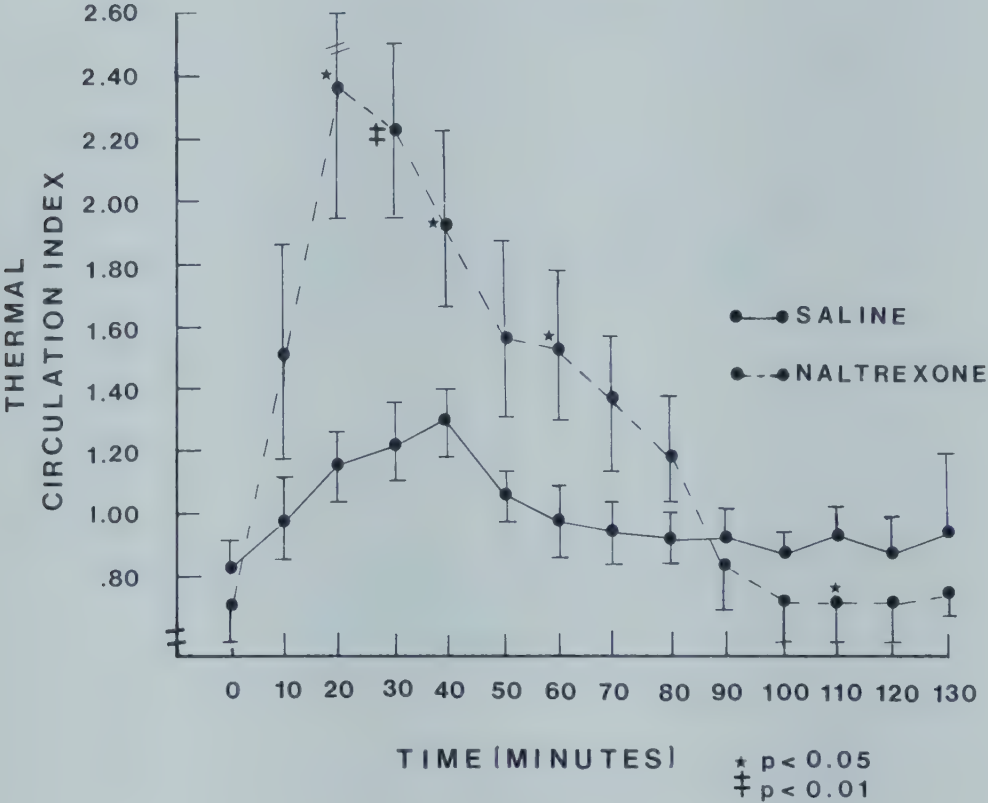


Figure 4. Mean thermal circulation index \pm S.E.M. over a 2-hour period of rats injected with either saline or 10 mg/kg naltrexone.

of saline, suggesting the recruitment of peripheral vessel dilation as an important 'vessel component' of thermoregulation. This is supported by the significantly elevated thermal circulation index (i) observed following the administration of the drug. Changes in the thermal circulation index are thought to reflect thermogenic vessel dilation in humans and other animals (15, 16). Therefore, the fact that i was higher when animals were injected with naltrexone than following saline injections, suggests that naltrexone produced vessel dilation in addition to the effects of the ip injection per se. The particular mechanism of this action needs further investigation.

Despite the significant differences in core temperature observed following saline and naltrexone injections in the present study, no significant differences were found in thermal conductance. Thermal conductance is a reflection of the organism's ability to lose heat, and therefore it is inversely correlated with insulation. Thermal conductance is in large part a reflection of the animal's adaptations against heat loss by such means as increased subcutaneous fat, increased fur and decreased peripheral circulation. The absence of a significant difference in thermal conductance after either saline or naltrexone administration, was due in part to the significant

suppression in oxygen consumption observed following naltrexone administration. Thus even though there was a lowered core temperature following naltrexone administration, the ratio of heat production to core temperature was maintained in both groups. The mechanisms responsible for the suppression of heat production in response to acute naltrexone administration and the ensuing hypothermia remain unclear.

In contrast to the present study, in a previous study in our laboratory in which we examined the effects of chronic infusions of naltrexone on Sucrose + chow-fed and Chow-fed animals, Sucrose + chow-fed animals exhibited increased oxygen consumption, increased CO_2 production and hyperthermia relative to Chow-fed controls (12). These differences were most likely due to the length of time animals were given access to their respective diets. In the previous study animals were maintained on the sucrose diet for only 3 weeks prior to observing the differences between groups. In contrast, in the present study, animals were given access to sucrose for a 3-month period. Therefore, these differences were most probably due to time-dependent adaptations to the sucrose-induced hyperphagia. It is interesting to note however, that chronic infusions of naltrexone via osmotic minipumps, also resulted in a hypothermia similar to that observed in the present study (12). In addition, chronic naltrexone infusions resulted in a significant suppression in CO_2 production. However, in contrast to the acute injections of naltrexone, chronic infusions did not decrease O_2 consumption. Therefore, as CO_2 production was suppressed while O_2 consumption wasn't altered, RQ was found to be significantly decreased following chronic administration.

Acute naltrexone administration, at ambient temperature (23-25°C), resulted in decreased oxygen consumption, decreased carbon dioxide production and a decrease in core temperature accompanied by a modification in the 'vessel' component of thermoregulation. Vasoconstriction did not accompany the significant decrease in core temperature as might be expected. Rather, the drop in core temperature was accompanied by a pronounced vasodilation. Therefore, animals were dropping their core temperature by peripheral vessel dilation, without compensating by increasing their heat production.

The present study provides evidence that naltrexone, at the tested dose, altered the thermal set point and suppressed heat production. As naltrexone is thought to have its effects by blocking the endogenous opioid system, the present study suggests a role for the opioid peptides in short term thermoregulation.

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REFERENCES

1. Clark, W. G. Effects of opioid peptides on thermoregulation. *Fedh. Proc.* 40, 2754 (1981).
2. Rosow, C.E., Miller, J.M., Poulsen-Burke, J. and Cochin J. Opiates and thermoregulation in mice. II. Effects of opiate antagonists. *J. Pharmacol. exp. Ther.* 220, 464 (1982).
3. Clark, W.G. Influence of opioids on central thermoregulatory function. *Pharmacol. Biochem. Behav.* 106, 609 (1979).
4. Numan, R. and Lal, H. Effect of morphine on rectal temperature after acute and chronic treatment in the rat. *Prog. Neuro-Psychopharmacol.* 5, 363 (1981).
5. Lotti, V.J., Lomax, P. and George, R. Temperature responses in the rat following intracerebral microinjection of morphine. *J. Pharmac. exp. Ther.* 150, 135 (1965).
6. Wong, D.L. and Bentley, G.A. The effect of morphine pretreatment on hypothermia induced by morphine in mice. *Eur. J. Pharmac.* 53, 391 (1979).
7. Cox, B., Ary, M., Chesarek, W. and Lomax, P. Morphine hyperthermia in the rat: An action on the central thermostats. *Eur. J. Pharmac.* 36, 33 (1976).
8. McGilliard, K.L., Tulunay, F.C. and Takemori, A.E. Antagonism by naloxone of morphine- and pentazocine-induced respiratory depression and analgesia and morphine-induced hyperthermia. In: *Opiates and Endogenous Peptides*, (H.W. Kosterlitz, editor), Amsterdam: Elsevier/North Holland 1976 p. 281.
9. Cowan, A. and MacFarlane, I.R. Effect of morphine antagonists on drug-induced hypothermia in mice and rats. *Psychopharmacology (Berlin)* 45, 277 (1976).
10. Mandenoff, A., Fumeron, F., Apfelbaum, M. and Margules, D.L. Endogenous opioids and energy balance. *Science* 215, 1536 (1982).
11. Margules, D.L., Moisset, B., Lewis, M.J, Shibuya, H. and Pert, C.B. B-endorphin is associated with overeating in genetically obese mice (ob/ob) and rats (fa/fa). *Science* 202, 988 (1978).
12. Marks-Kaufman, R., Balmagiya, T. and Gross, E. Modifications in food intake and energy metabolism in rats as a function of chronic naltrexone infusions. *Pharmacol. Biochem. Behav.* 20, 911 (1984).
13. Kanarek, R. B. and Marks-Kaufman, R. Developmental aspects of sucrose-induced obesity in rats. *Physiol. Behav.* 23, 881 (1979).
14. Aschoff, J. Thermal conductance in mammals and birds: its

dependence on body size and circadian phase. *Comp. Biochem. Physiol.* 69A, 611 (1981).

15. Richards, S.A. *Temperature Regulation*, Springer-Verlag, New York, 1973.

16. Balmagiya, T. and Rozovski, S.J. Age-related changes in thermoregulation in male albino rats. *Experimental Gerontology* 18, 199 (1983).

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CHANGES IN PHYTATE AND RELATED COMPOUNDS IN GRAIN SORGHUM DURING GERMINATION

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ABSTRACT

Changes in phytate and related compounds in Sorghum bicolor grain were monitored over a 10 day germination period. Germinated sorghum is used as malt in the sorghum beer brewing industry and, hence, it is desirable to understand what changes occur during germination. The grain achieved a 97% germination rate and after 10 days the roots and shoots accounted for 20% of the dry weight. Ten days germination caused a 100% reduction in the phytate content while the percentage content of this mineral element either remained constant or increased. The lipid content decreased during the first half of the germination period then increased during the last half. As it is desirable to minimise the lipid contributed by the malt, a five to six day germination is optimal in this respect. Overall, germination improved the nutritional quality of the sorghum grain.

INTRODUCTION

Grain sorghum (Sorghum bicolor (L.) Moench) production is the fourth largest of all cereals worldwide and it is extensively used as a food. In Africa large quantities of sorghum are germinated each year to produce malt for the production of traditional sorghum beer. Because changes in the starchy endosperm during germination are indicators of malt quality, this aspect has been well studied in sorghum (1,2,3). However, little is known of the changes that occur in other tissues of the grain. The purpose of this work was to examine some nutritionally important components of the aleurone layer and germ.

Phytate plays an important role in the storage of mineral elements in cereal grains (4). Phytate is 1,2,3,4,5,6 hexakis (dihydrogen phosphate) myo-inositol which is the major form of phosphorus storage in cereal grains and it usually occurs as the mixed calcium, magnesium, potassium and zinc salt. Because phytate reduces the bioavailability of important mineral elements, it is essential to understand any process such as germination which would affect its activity. In conjunction with the phytate content, it is important to study the mineral element content of the grain during germination.

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Lipids have previously been reported from sorghum grain (5). If these lipids are carried over into the beer in sufficient quantity, then such factors as foam quality and beer staling can be affected. There is a significant decrease in the lipid content during the germination of barley (6) and it was decided to determine if a similar decline occurred in sorghum.

MATERIALS AND METHODS

A sample of sorghum grain, Cultivar NK 283, was obtained from a commercial source and germinated according to methods already described (7). Samples were taken each day for 10 days and dried at 50° for 24 h in a forced-air oven, after which they contained approximately 6% moisture. Samples for transmission electron microscopy were prepared by standard techniques (2).

Before the germinated kernels were analysed, the roots and shoots were first removed. This was accomplished by rubbing the dried seedlings in a nylon bag with a mesh size that allowed the roots and shoots to escape while retaining the kernels. After separation, the kernels and the roots and shoots were milled separately for 1 min in a Janke and Kunkel mill (a water cooled beater type mill).

Phytate was separated after extraction with 3% aqueous trichloroacetic acid by HPLC and quantitated as phytic acid by an in-line post column colorimetric reaction (8). For lipid determination a 5 g sample was Soxhlet extracted with hexane for 6 h. The final extract was evaporated on a rotary evaporator and over dried at 100° for 2 h before being weighed. Mineral elements were determined by atomic absorption after the samples were dry ashed at 520°.

RESULTS AND DISCUSSION

A germination count revealed that after 24 h the germination rate was 64% and the full germination rate of 97% was reached at 72 h.

Phytate was greatly reduced during the 10 day germination period used in this study (Figure 1). The phytate decreased from a value of 160 mg per 1,000 kernels at time of steeping to a zero value after 10 days germination. From a nutritional point of view phytin is troublesome as it is capable of interfering with the utilization of mineral elements and phosphorus uptake in humans and monogastric animals (9). Since sorghum is used to produce malt for sorghum beer brewing, the phytate content could present a problem. However, germination has been found to cause a reduction in phytate in both cereals and legumes (10,11).

The phytate in the sorghum kernel declined during germination until it was completely degraded after 10 days (Figure 1). However, for the production of sorghum malt the grain is germinated for only six days. From Figure 1 it can be seen that there was approximately a 75% decrease in phytate during this period.

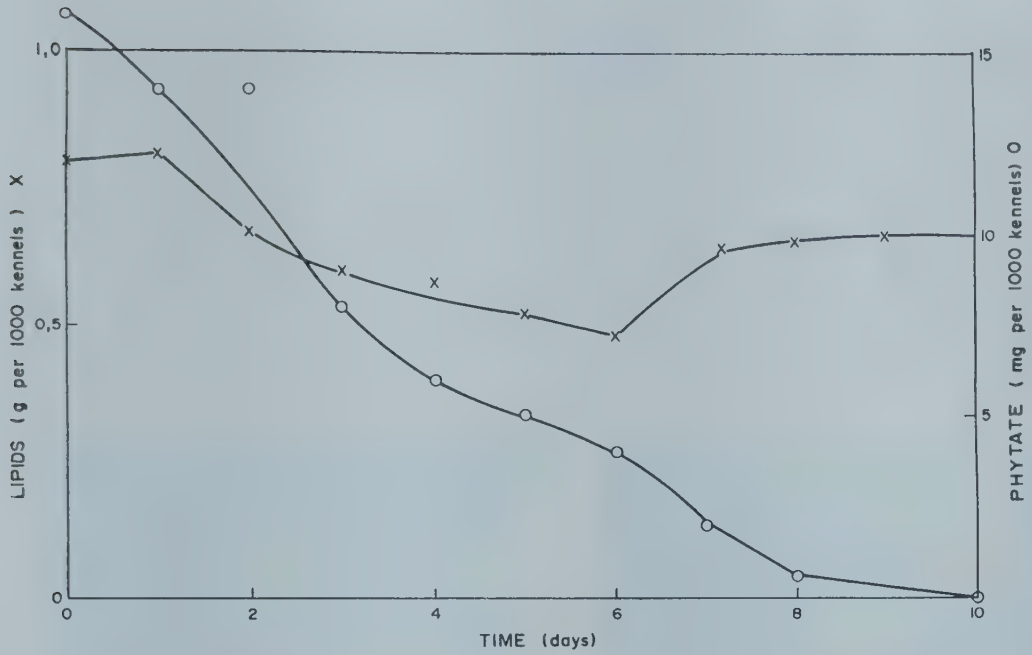


FIG. 1. Lipid and phytate content in sorghum grain germinated for different times.

To improve our understanding of the germination process and to follow the physical changes which occur during this period, samples

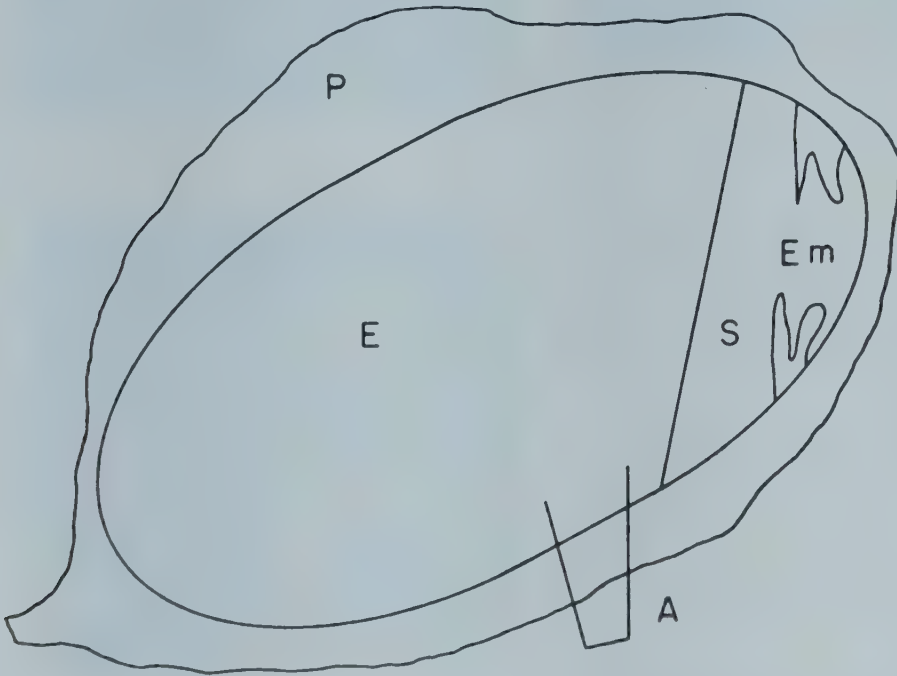


FIG. 2. Approximate location (A) in kernel where sections were taken for electron micrographs.
P = pericarp; E = endosperm; S = scutellum; Em = embryo.

of the germinating grain were fixed in gluteraldehyde and prepared for electron microscopy. The location in the germinating kernel from which sections were taken, is shown in Figure 2. Sections from the grains of different germination periods were taken from the same location in an effort to make them comparable.

A study combining chemical analysis with electron microscopic observation, allows the chemical changes to be related to the structural modification. Figures 3 to 5 show transmission electron micrographs of the aleurone cells after different periods of germination. After steeping, the aleurone cells appear to be reasonably complete (Figure 3). The lipid bodies continue to surround the periphery of the cells as well as the aleurone grains.

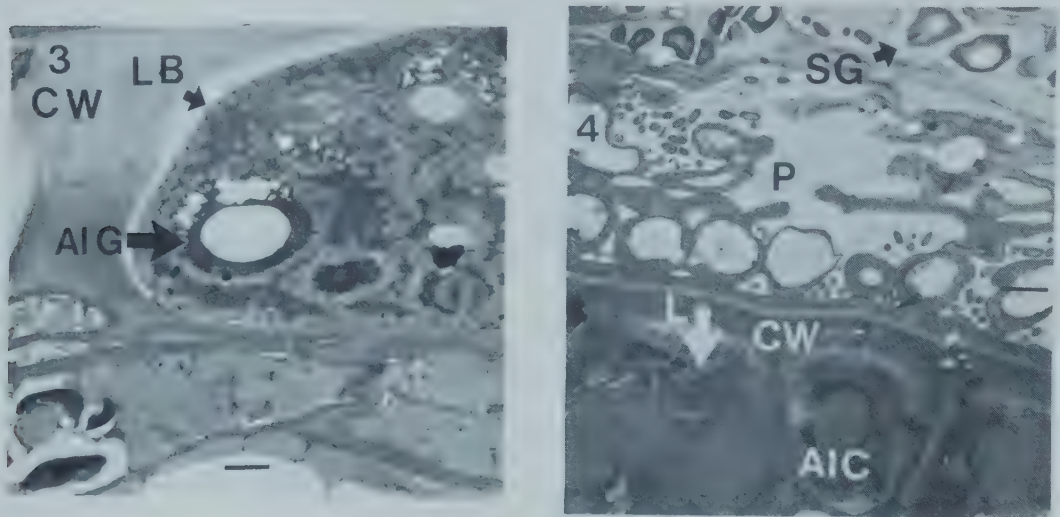
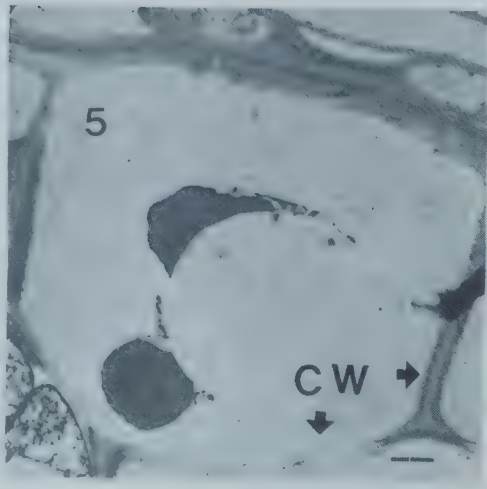


FIG. 3. Transmission electron micrograph of aleurone cell in sorghum grain steeped for 24 h. Al G = aleurone grain; CW = cell wall; LB = lipid body. Bar = 1.3 μ m.

FIG. 4. Transmission electron micrograph of aleurone cells and sorghum grain germinated for six days. Al C = Aleurone cell; CW = cell wall; LB= lipid body; P = pericarp; SG = Starch granule. Bar = 3.2 μ m.

FIG. 5. Transmission electron micrograph of aleurone cell of sorghum grain germinated for 10 days. Note absence of cell contents. CW = cell wall. Bar = 2.0 μ m.



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After six days germination the phytate level was reduced to 75% that found in the dormant grains (Figure 1). The aleurone grains, in which the phytate is stored, lost their individual structure and were coalesced into very few bodies per cell (Figure 4). Also, the number of lipid bodies has been greatly reduced as can be seen both from the micrograph (Figure 4) and from the chemical analysis (Figure 1). The ultrastructural changes described here for sorghum parallel those observed in the aleurone cells of barley (12). These changes are not reflected in the pericarp where the small starch granules appear to be completely intact (Figure 4).

The aleurone cells after 10 days germination appeared virtually empty (Figure 5). No discernible structures remained, only small fragments of apparent debris. However, the cell walls appeared to be virtually intact except for those adjoining the starchy endosperm. The phytate content as expressed in Figure 1 would agree with this observation but the lipid content certainly would not.

The lipid content of the sorghum kernels during germination is shown in Figure 1. The hexane soluble lipids declined during the first six days of germination and subsequently increased until after 10 days germination they were more than double the original content. A similar pattern of decline then increase was reported for both wheat and maize (13) but the time scale was shorter. As can be seen from the micrographs, there is a decline in lipid bodies in the aleurone layer throughout this study (Figures 3,4,5). However chemical analysis has shown an increase in lipid during the later part of the germination period. One possible explanation is that the lipid is present in the scutellum. The lipid in the scutellum could be a temporary form of storage. Starch in the endosperm was hydrolysed during germination and the resultant sugar transported to the scutellum. If the embryo does not immediately require this carbohydrate then it must be stored and lipid could provide a simple form of storage.

Usually associated with phytate are mineral cations and in Table 1 the cation content of the grain over nine days of germination is listed. There appears to be a reasonably large increase in Ca ions while the Mg and Fe increased only slightly. The Cu, Zn and Mn showed no increase. P showed an increase and it should be noted that P was determined after ashing so that total P, both organic and inorganic, is included in this figure. Also, it should be emphasized that these results are for the germinated kernel and do not include the roots and shoots. As expected, the kernels lost weight hence this could enhance the mineral element content on a percentage basis.

During germination the mineral elements in the phytate of the aleurone grains progressively migrate to the developing seedling. However, the grain used in these experiments were steeped twice a day in tap water for a total daily time of 15 min. Therefore, the grain could pick up mineral elements from the tap water to account for the increase shown in Table 1. Minerals usually move from the kernel to the growing embryo and yet the mineral content of the kernel remained static or increased. Besides the loss in kernel weight,

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TABLE 1. Element content in sorghum kernels germinated for different times.

Days Germination	Mineral Element (mg/100 g)						
	Ca	Mg	Cu	Fe	Zn	Mn	P
0	9.0	123.0	0.31	ND	1.83	1.28	201
S(a)	37.6	137.3	0.30	3.10	1.90	1.46	233
1	36.5	138.8	0.23	3.11	1.97	1.29	232
2	43.2	144.6	0.27	3.07	1.67	1.19	234
3	43.5	156.6	0.20	3.07	1.91	1.32	239
4	47.1	144.7	0.26	3.01	1.82	1.37	232
5	53.1	142.6	0.30	ND	2.00	1.37	249
6	57.8	164.6	0.32	3.21	1.80	1.45	254
7	56.0	142.6	0.32	3.36	1.95	1.43	236
8	62.7	150.8	0.45	3.95	1.85	1.39	258
9	68.7	147.4	0.44	5.07	1.84	1.32	281

(a) - Steeped

ND - Not Determined

the mineral element increase could also be explained by an uptake of minerals during steeping. This problem could have been overcome by steeping the grain in distilled water. However, this idea was rejected as under practical conditions the grain would not be steeped in distilled water.

CONCLUSIONS

Germination of sorghum grain caused major changes in the chemical composition of the aleurone layer. However, the pericarp appeared to be untouched by enzyme action.

The choice of a six day germination period under current conditions for the production of sorghum malt, appears to be a wise one. After six days germination the lipid content of the kernel appears to be at its lowest level. The nutritional quality of the grain appears to be improved by germination. There was a reduction in the phytate content while the content of some of the mineral elements increased.

REFERENCES

1. Aisien, A.O. Enzyme modification of sorghum endosperm during seedling growth and malting. *J. Sci. Food Agric.* **33**, 754 (1982).
2. Glennie, C.W., Harris, J. and Liebenberg, N.v.d.W. Endosperm modification in germinating sorghum grain. *Cereal Chem.*

NUTRITION REPORTS INTERNATIONAL

60, 27 (1983).

3. Taylor, J.R.N. Effects of malting on the protein and free amino nitrogen composition of sorghum. *J. Sci. Food Agric.* 34, 885 (1983).
4. Eastwood, D. and Lairdman, D.L. The mobilization of macro-nutrient elements in the germinating wheat grain. *Phytochemistry* 10, 1275 (1971).
5. Deyoe, C.W. and Robinson, R.J. Sorghum and pearl millet foods. In: *Tropic Foods*, eds. Inglet, G.E. and Charalambous, G. Vol. 1. Academic Press, New York. (1979).
6. Palmer, G.H. and Bathgate, G.N. Malting and brewing. In: *Advances in Cereal Science and Technology*. Ed. Pomeranz, Y. Vol. 1. American Assoc. Cereal Chem., St. Paul, USA, (1976).
7. Glennie, C.W. Endosperm cell wall modification in sorghum grain during germination. *Cereal Chem.* 61, 285 (1984).
8. Cilliers, J.J.L. and van Niekerk, P.J. LC determination of phytic acid in foods by post-column colorimetric detection. *J. Agric. Food Sci.*, (submitted).
9. Ologhobo, A.D. and Fetuga, B.A. Distribution of phosphorus and phytate in some Nigerian varieties of legumes and some effects of processing. *J. Food Sci.* 49, 199 (1984).
10. Clutterbuck, V.J. and Briggs, D.E. Phosphate mobilization in grains of *Hordeum distichon*. *Phytochemistry* 13, 45 (1974).
11. Eskin, N.A.M. and Wiebe, S. Changes in phytase activity and phytate during germination of two faba bean cultivars. *J. Food Sci.* 48, 270 (1983).
12. Gram, N.H. The ultrastructure of germinating barley seeds. 1. Changes in scutellum and the aleurone layer in nortal barley. *Carlsberg Research Comm.* 47, 143 (1982).
13. Bolling, H. and El Baya, A.W. Changes in lipid composition during the germination and development of grain. In: *Development in Food Science*, eds. Holas, J. and Kratochvil, J. Vol. 5A. Elsevier, Amsterdam. (1983).

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PROTEIN AND NUCLEIC ACID METABOLISM IN THE LIVER OF FEMALE RATS DURING GRADED DIETARY RESTRICTION

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ABSTRACT

Three-week-old female rats were exposed to 10%, 30% or 50% dietary restriction for periods of 3, 9, 15 or 24 weeks and injected with ^{14}C -leucine or ^3H -orotate. Body weight, hepatic weight, total RNA, DNA and protein content of liver failed to increase normally in the various experimental groups. However, total free nucleotides and amino acids as well as their respective specific activities showed significant decreases. Incorporation of ^3H -orotate per mg RNA was diminished at 3 and 9 weeks, but increased markedly at 15 and 24 weeks. ^{14}C -leucine-incorporation per mg protein generally rose progressively on 10% and 30% dietary restriction with a decline at all time intervals in animals subjected to 50% food deprivation. The differences between the control and experimental groups were similar from 3 to 24 weeks, indicating a consistent effect of graded dietary restriction for the duration of the experiment.

INTRODUCTION

Research into the role of diet in growth, aging and lifespan of laboratory animals was first started in the earlier part of this century, by McCay and his co-workers (1-4). Since then, much interest has been generated toward understanding the phenomena of extended lifespan and decreased susceptibility to disease among animals whose growth has been retarded by dietary manipulation.

The rat seems to be the species least stunted permanently by prolonged growth retardation, provided that food restriction is not initiated until after the weaning period. McCay *et al.* (3) reported a favorable effect of growth inhibition for up to 900 days, leading to the generation of very old rats. The maturation of both males and females is slowed by dietary manipulation but the viability of sperm and continuation of the estrus cycle persist until much later than normal. Growth-retarded rats

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exhibit much greater resistance to chronic diseases than normal animals, although these diseases do eventually occur (3,5,6).

In recent years, many researchers have focused their attention on the influence of dietary restriction upon the synthesis and content of various biochemical components (7-16). These investigations have revealed a general depletion of protein, RNA and DNA in several organs, as a result of either their diminished synthesis or augmented activities of one or more enzymes involved in their respective metabolism.

On the basis of these interesting studies, it seemed worthwhile to examine the effects of varying degrees of food deprivation on growth, metabolic liver function and aging in young rats to shed some light on the question of how the organism responds to nutritional stress in different phases of active growth.

MATERIALS AND METHODS

Experimental protocol. Sixty-four white female Sprague-Dawley rats (Canadian Breeding Farms, St. Constant, Quebec), aged 3 weeks and weighing 45 ± 5 g, were divided into 4 equal groups. Each group was further divided into 4 equal subgroups, studied at time intervals of 3, 9, 15 and 24 weeks, respectively.

The controls (C) were fed a specially-constituted, pellet-form diet (Teklad Test Diets, Madison, Wisconsin) ad libitum. Their average daily consumption was calculated and employed as the basis for the quantity of food given to the 3 experimental groups (Table I). Experimental Groups E1 and E2 respectively received 90% and 70% of the diet consumed on the previous day by Group C. Experimental Group E3 was given 50% of the quantity consumed by Group C on the previous day (i.e., 50% dietary restriction), but received a different diet (Teklad), which included increased vitamins and minerals to offset the dietary restriction and to eliminate the possibility of metabolic changes due to vitamin- and mineral-deficiency. All the rats were weighed once a week during the experimental period.

In another series of experiments, normal animals were allowed to grow to the same body weight as the E3 group at the time the latter rats were killed. This study was undertaken to compare liver weight as well as RNA, DNA and protein content of the E3 population with corresponding values of the younger controls.

Preparation of samples for analysis of DNA, RNA, proteins and radioactivity. Four hours before being sacrificed at the end of the designated time intervals of 3,

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Table I. Composition of diet fed to control and experimental animals

<u>Diet No. 1 (with 7.5% cellulose)¹</u>	
<u>Ingredients</u>	<u>Amount (g)</u>
Casein (Vitamin-free test)	26.0
Sucrose	43.0
Corn starch	15.0
Corn oil	4.0
Non-nutritive fibre (cellulose)	7.5
Mineral mix (Williams-Briggs modified) ²	3.5
Vitamin mix (Teklad) ³	1.0
Total	100.0
<u>Diet No. 2 (with 3.0% cellulose and increased minerals and vitamins)⁴</u>	
Casein (Vitamin-free test)	26.0
Sucrose	43.0
Corn starch	15.0
Corn oil	4.0
Non-nutritive fibre (cellulose)	3.0
Mineral mix (Williams-Briggs modified)	7.0
Vitamin mix (Teklad)	2.0
Total	100.0

¹Given to the controls and Groups E1 and E2.

²Percent composition: CaCO₃ 20.714; CaHPO₄ 32.285; CuSO₄ 0.037; FeC₆H₅O₇ 5H₂O 0.431; MgSO₄ 6.571; MnSO₄ H₂O 0.440; KCl 20.857; KIO₃ 0.002; Na₂HPO₄ 18.6; ZnCO₃ 0.060. Teklad Test Diet Catalog #170911.

³Supplies in g or units per kg of diet: p-aminobenzoic acid 110.13 mg; ascorbic acid, coated (97.51) 991.2 mg; biotin 0.441 mg; vitamin B₁₂ 0.0297 mg; calcium pantothenate 66.0 mg; choline dihydrogen citrate 1433.7 mg; folic acid 1.982 mg; inositol 110.13 mg; menadione 49.5 mg; niacin 99.1 mg; pyridoxine HCl 22.0 mg; riboflavin 22.0 mg; thiamine HCl 22.0 mg; dry vitamin A palmitate (500,000 U/g) 19,824 units; dry vitamin D₂ (500,000 U/g) 2,202.5 units; dry vitamin E acetate (500 U/g) 121.15 units; corn starch 4666.878 mg. Teklad Test Diet Catalog #40060.

⁴Fed to Group E3.

9, 15 and 24 weeks, each animal received an intraperitoneal injection of ¹⁴C-leucine (specific activity 251.0 mCi/mmol, concentration 0.0523 mg in 1 ml solution containing 0.1 mCi)

or ^3H -orotate (specific activity 41.7 mCi/mmol, concentration 0.417 mg in 0.1 mCi) at dose levels of 2.5 $\mu\text{Ci}/50\text{ g}$ body weight. Their livers were isolated, weighed, maintained at 0°C , and homogenized as 1-g samples in a Polytron Model 103 homogenizer (Talboys Instrument Corp., Toronto, Ontario).

RNA was extracted and quantified by the procedure of Munro and Fleck (17), using alkaline hydrolysis. DNA was assayed by the diphenylamine reaction as modified by Burton (18). Proteins in each sample were determined by Goa's technique (19). In the incorporation studies, proteins were precipitated with 5% trichloroacetic acid (TCA) and kept for 2 hours at 0°C . They were then filtered via a Millipore filtration apparatus and washed with 95% ethanol and ether. The dried protein and filter paper were subsequently transferred to scintillation vials and dissolved in 1 ml NCSTM solubilizer (Nuclear Chicago-Searle, Toronto, Ontario). After adding 14 ml of diluted liquifluor (POPOP), the whole solution was shaken vigorously and the vials left overnight in a refrigerator for counting the next morning in a Nuclear Chicago Mark II scintillation counter. For the incorporation of ^3H -orotate into RNA, supernatant containing the hydrolyzed RNA (17) was mixed with Aquasol and counted as described above. Studies on the specific activity of the pool size of free amino acids and total free nucleotides were accomplished by combining the supernatant containing these constituents (17) with Aquasol and counting them as outlined above.

Calculations and statistical analysis. The optical density readings (% transmission) of each of the three components (DNA, RNA and protein) at the specified wavelengths on the Perkin-Elmer double-beam spectrophotometer (Coleman 124) were compared to the values on each of the respective standard curves. Each value thus obtained was correlated mathematically to the volume of homogenate used in the particular experiment, taking into account the dilution factor, then extrapolated to the quantity of tissue and, finally, to the entire organ weight. The quantified specific component was thus correlated to the whole liver. The free amino acid pool size was determined by relating optical density to the volume of the supernatants and extrapolating it to the g organ weight. Free nucleotide calculations were based on the optical density of a standard (2 mg%) solution of nucleotides and correlated to each g of tissue by mathematical manipulation. Specific activities were then calculated for each of the cellular components under study.

The Student's t-test was performed on each set of results to obtain the significance of the difference between two population means.

RESULTS

The body and liver weights of Groups E1, E2 and E3 generally failed to reach the normal levels of Group C in proportion to the degree of dietary restriction (Table II). At the same time, the experimental animals showed relative decreases in the liver weight to body weight ratio with some exceptions which were not statistically significant (Group E1 early in the study).

Graded dietary restriction elicited a progressively lower RNA content per total liver and affected cell growth by demonstrating a reduced cell population (hypoplasia) with diminished DNA levels relative to the degree of food deprivation (Table II). Consequently, although cellular weight increased in the 3 experimental groups from 3 to 9 weeks, it tended to decrease at 15 weeks and to a greater extent at 24 weeks in E2 animals, while showing augmented values in Groups E1 and E3 at the last autopsy.

The protein content in each of the experimental groups diminished progressively from Group C to E3 animals at all time intervals (Table III). An appreciable fluctuation was noted in the accumulation of dietary protein, as evidenced by the protein:DNA ratio.

There was a progressive decrease in the size of the free amino acid pool and the total free nucleotide content in the different experimental groups in comparison to Group C (Table III).

Metabolic activity of the cellular constituents was decidedly altered in the face of graded dietary restriction. Changes were evident in the specific activities of free amino acids and nucleotides as well as in the incorporation of labelled leucine into proteins and per mg RNA and of labelled orotate into RNA and per mg DNA (Table IV). However, the eventual situation was one of diminished competence of metabolic function in the experimental groups relative to the controls. The observed alterations were usually in line with the degree of dietary restriction imposed (Table IV).

DISCUSSION

The results of the present study clearly demonstrate a marked dependence of body and liver growth on the quantity of food consumed. The consistent nature of these effects coincides with observations reported by other investigators (7-16,20), who conducted similar experiments on the influence of dietary restriction upon organ systems and concluded that severe growth retardation accompanies malnutrition in young animals and children.

Table II. Effect of graded dietary restriction on body and liver weight, RNA and DNA content and liver cell weight at various time intervals

Time Intervals (weeks)	Group	Body weight (g)	Liver weight (g)	Total RNA (mg)	RNA:DNA	Total DNA	Liver cell weight (organ weight: DNA ratio)
3	C	162 + 3 ¹	5.04 + 0.05	44 + 2	1.92 + 0.08	23 + 1	1120 + 76
	E1	151 + 3 ^a	5.05 + 0.23	43 + 3	3.02 + 0.32 ^a	20 + 3	1578 + 82 ^a
	E2	117 + 3 ^b	3.03 + 0.01 ^a	31 + 1 ^{a,b}	2.95 + 0.18 ^b	18 + 2 ^a	875 + 80 ^{a,b}
	E3	94 + 2 ^b	2.28 + 0.28 ^{a,b}	19 + 3 ^{a,b}	2.23 + 0.40 ^a	14 + 2 ^a	640 + 140 ^{a,b}
9	C	250 + 8	6.28 + 0.09	48 + 3	1.68 + 0.23	30 + 2	1243 + 64
	E1	232 + 4 ^a	6.78 + 0.50	51 + 4	2.28 + 0.12 ^a	23 + 2	2209 + 92 ^b
	E2	187 + 3 ^b	5.38 + 0.17 ^{a,b}	36 + 4 ^a	2.61 + 0.33 ^a	18 + 3 ^a	2228 + 537 ^a
	E3	148 + 4 ^b	3.65 + 0.09 ^{a,b}	27 + 2 ^{a,b}	2.48 + 0.23 ^a	11 + 1 ^{a,b}	1252 + 134 ^{a,b}
15	C	238 + 17	6.98 + 0.69	63 + 4	2.60 + 0.30	26 + 5	1948 + 191
	E1	231 + 1	6.52 + 0.57	47 + 6 ^a	1.93 + 0.19 ^a	24 + 1	1520 + 108 ^a
	E2	198 + 3 ^{a,b}	4.38 + 0.05 ^{a,b}	35 + 1 ^{a,b}	2.19 + 0.19	16 + 2 ^a	1220 + 132 ^a
	E3	158 + 2 ^b	3.58 + 0.06 ^b	28 + 1 ^{a,b}	1.48 + 0.14 ^a	19 + 1 ^a	677 + 50 ^b
24	C	290 + 8	6.98 + 0.20	45 + 2	1.93 + 0.35	26 + 2	1912 + 104
	E1	253 + 10 ^a	6.53 + 0.06	47 + 3	2.49 + 0.20	19 + 1	2304 + 163 ^a
	E2	209 + 4 ^b	4.78 + 0.14 ^b	31 + 2 ^b	1.47 + 0.13 ^b	21 + 1 ^a	1086 + 74 ^b
	E3	164 + 4 ^b	3.48 + 0.08 ^b	22 + 1 ^b	1.58 + 0.11 ^{a,b}	14 + 1 ^{a,b}	896 + 72 ^{a,b}

¹Each value is the average of 4 animals (Mean + S.E.).
^ap<0.05 as compared to C, E1, E2 or all 3 groups.
^bp<0.005 as compared to C, E1, E2 or all 3 groups.

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Table III. Effect of graded dietary restriction on protein content, protein:DNA ratio, free amino acid and total nucleotide pool size at various time intervals

Time intervals (weeks)	Group	Total protein (mg)	Protein:DNA	Free amino acid pool size (absorption at 280 nm)	Free nucleotide content (mg)
3	C	1340 \pm 50 ¹	59 \pm 3	201 \pm 1	106 \pm 2
	E1	1200 \pm 130	86 \pm 13 ^a	202 \pm 14	102 \pm 10
	E2	630 \pm 10 ^a	60 \pm 8 ^a	224 \pm 0.5 ^b	39 \pm 3 ^b
	E3	450 \pm 30 ^{a,b}	54 \pm 6 ^a	182 \pm 20 ^a	34 \pm 8 ^b
9	C	1325 \pm 75	56 \pm 6	292 \pm 19	84 \pm 2
	E1	1195 \pm 35	54 \pm 1	354 \pm 26 ^a	105 \pm 6 ^a
	E2	920 \pm 75 ^a	68 \pm 13	236 \pm 26 ^a	80 \pm 4 ^a
	E3	670 \pm 30 ^{a,b}	63 \pm 6	174 \pm 4 ^{a,b}	56 \pm 5 ^{a,b}
15	C	1950 \pm 150	70 \pm 11	242 \pm 25	119 \pm 8
	E1	1550 \pm 80 ^a	65 \pm 6	224 \pm 19	113 \pm 9
	E2	1020 \pm 40 ^b	65 \pm 7	170 \pm 13 ^a	100 \pm 4 ^a
	E3	940 \pm 30 ^{a,b}	50 \pm 2 ^a	153 \pm 8 ^a	89 \pm 3 ^a
24	C	1700 \pm 110	66 \pm 17	324 \pm 18	183 \pm 17
	E1	1490 \pm 70 ^a	80 \pm 4 ^a	312 \pm 11	169 \pm 5
	E2	1120 \pm 40 ^b	53 \pm 3 ^{a,b}	220 \pm 9 ^b	130 \pm 8 ^{a,b}
	E3	910 \pm 30 ^{a,b}	68 \pm 7 ^a	152 \pm 8 ^b	83 \pm 2 ^b

¹ Each value is the average of 4 animals (Mean \pm S.E.).

^a $p < 0.05$ as compared to C, E1, E2 or all 3 groups.

^b $p < 0.005$ as compared to C, E1, E2 or all 3 groups.

The rate of accumulation of such cellular constituents as RNA, DNA and protein is generally hampered in proportion to the degree of food deprivation. Changes in the accumulation of RNA versus DNA during the first 9 weeks of the experiment indicate diminishing hyperplasia with increasing hypertrophy, as suggested by a re-direction of nucleotides away from DNA synthesis toward RNA synthesis. The increases in cell weight and decreases in cell number in Groups E1, E2 and E3 from 3 to 9 weeks support this conclusion. The present data on the chaotic nature of protein synthesis in the first two time intervals concur with those of Winick *et al.* (15) and Srivastava *et al.* (10,11), who believe that alterations in ribosomal RNA metabolism or t-RNA activity indirectly affect protein production. A clearer pattern of organ growth emerges at 15 and 24 weeks: from the ratios of RNA:DNA and protein:DNA, it becomes evident that intracellular RNA and subsequently protein are lost at increasingly higher rates, that is, catabolism exceeds anabolism. The process of cell

Table IV. Effect of graded dietary restriction on protein and RNA synthesis in the rat liver

Time intervals (weeks)	Group	¹⁴ C-leucine			³ H-Orotate		
		Specific activity of free amino acids (dpm/optical density at 280 nm)	Protein synthesis (dpm/mg protein)	Incorporation/unit ribosome (dpm/mg RNA)	Specific activity of free nucleotides (dpm/mg total nucleotides)	RNA synthesis (dpm/mg RNA)	RNA incorporation (dpm/mg DNA)
3	C		510 ± 50 ¹	15635 ± 1283		9689 ± 686	16829 ± 1062
	E1		524 ± 48	14342 ± 396		8839 ± 246	24011 ± 2192 ^a
	E2		654 ± 82	13381 ± 2619		8433 ± 1544	25173 ± 6055 ^a
	E3		420 ± 52 ^a	10309 ± 690 ^{a,b}		7125 ± 200 ^{a,b}	21734 ± 970 ^a
9	C		604 ± 88	17928 ± 1074		10142 ± 440	15690 ± 1309
	E1		616 ± 64	14652 ± 2072		9580 ± 1260	16074 ± 1674
	E2		644 ± 64	15784 ± 386 ^a		9772 ± 398	22776 ± 2517 ^a
	E3		540 ± 32	13854 ± 1294 ^a		8438 ± 708 ^a	18302 ± 123 ^a
15	C	4149 ± 202	468 ± 16	14531 ± 402	4775 ± 365	10721 ± 473	24683 ± 1987
	E1	4673 ± 520	604 ± 40 ^a	20933 ± 2710 ^a	4734 ± 450	15004 ± 1898 ^a	24125 ± 1726
	E2	5011 ± 416 ^a	772 ± 30 ^{a,b}	22604 ± 819 ^b	5064 ± 270	16368 ± 704 ^b	32926 ± 2720 ^a
	E3	3769 ± 360 ^a	586 ± 30 ^{a,b}	20144 ± 1592 ^a	3897 ± 378 ^a	14514 ± 1510 ^a	19368 ± 1938 ^a
24	C	3323 ± 194	434 ± 38	16331 ± 770	3205 ± 302	10519 ± 783	22878 ± 2673
	E1	3219 ± 85	486 ± 8	15736 ± 614	2969 ± 123	11039 ± 302	22483 ± 1821
	E2	3971 ± 156 ^{a,b}	570 ± 24 ^a	21031 ± 1496 ^a	3449 ± 84 ^a	14143 ± 1018 ^a	20403 ± 977 ^a
	E3	3425 ± 207 ^a	364 ± 54 ^a	15642 ± 1661 ^a	2930 ± 277 ^a	10873 ± 1234	16723 ± 546 ^{a,b}

¹Each value is the average of 4 animals (Mean ± S.E.).

^ap<0.05 as compared to C, E1, E2 or all 3 groups.

^bp<0.005 as compared to C, E1, E2 or all 3 groups.

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division is halted by the effects of malnutrition as an "adaptive response" to graded dietary restriction (8,21). The present results on the progressive decreases in the pool size of free amino acids and free nucleotides in the experimental groups are directly correlated to the effects of graded malnutrition (8).

The diminished specific activities of the free amino acid and nucleotide pools, the overall reduction in the synthesis of protein, coupled with the increased incorporation per unit ribosome in food-deprived animals, all point toward an accelerated metabolism of protein and RNA with an ultimate drop in cellular protein accumulation. This, together with the steady fall in RNA incorporation per cell, is in agreement with similar observations reported earlier (22).

The present data indicate an absolute metabolic retardation of Group E3 rats as a result of their dietary restriction. At no point did they surpass the chronologic age of 6.3 weeks in terms of maturation (Table V). Indeed, a plateau in their development was reached very early in life. The decreased cell population and augmented cell size observed in these animals would also suggest a permanence in the effects of severe undernutrition (50% of normal food consumption) to which they were subjected. If this is so, subsequent ad libitum feeding would only cause further cellular hypertrophy with no action upon hyperplasia but with concomitant effects on RNA and protein synthesis due to diminished cell numbers, as suggested by Jasper and Brasel (23).

It is possible that dietary restriction enhances certain enzyme systems which are responsible for health, while inhibiting those that exert degradative, aging activities. These food-deprived rats might somehow bypass physiologic maturity with its cumulative "morbid" effects on the organism. The gradual curtailment of DNA depression, postulated to occur in older animals (24) with its concomitant actions on the induction of genes and subsequent protein synthesis, would then be prevented. In addition, the organism could also adapt to much lower requirements of high-energy phosphate compounds (25) and the expected age-related decline in hormone responsiveness would thus be stalled.

In conclusion, it has been shown that the manifestations of undernutrition are proportionally related to the level of food intake in laboratory rats and become more pronounced over time. It would appear that food-deprived animals develop a new steady metabolic state, which diminishes their demands for more food and seems to stabilize after approximately $3\frac{1}{2}$ to 4 months of dietary restraint. Group E3 rats -- the most extreme result of the

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Table V. Age when control animals reached the values of experimental rats exposed to 50% dietary restriction

Duration of food restriction	3 weeks (42)*	9 weeks (84)	15 weeks (126)	24 weeks (189)
Parameter	Age (days)			
Body weight	28	38	41	44
Liver weight	17	30	30	30
RNA	16	25	25	19
DNA	23	18	32	23
Protein	13	20	29	28

*Figures in parentheses represent the chronologic age (in days) of the experimental animals at each time interval studied.

experiment -- behaved metabolically at 189 days (or 27 weeks) of age as if they were 20-44 days (3-6 weeks) old, that is, 10 to 23% of their chronologic age. If this observation is extended a little further, it could be postulated that these animals would live roughly four times longer than their littermates fed ad libitum. The patterns of development which were established correlate positively with the degree of dietary restraint and the length of time it was applied. These findings indicate biochemical parameters that are commensurate with a delayed aging process.

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REFERENCES

1. McCay, C.M., Crowell, M.F. and Maynard, L.A. The effect of retarded growth upon the length of lifespan and upon

NUTRITION REPORTS INTERNATIONAL

- the ultimate body size. J. Nutr. 10, 63 (1935)
2. McCay, C.M., Ellis, G.H., Barnes, L., Smith, C.A.H. and Sperling, G. Chemical and pathological changes in aging and after retarded growth. J. Nutr. 18, 15 (1939)
 3. McCay, C.M., Sperling, G. and Barnes, L.L. Growth, aging, chronic diseases and lifespan in rats. Arch. Biochem. 2, 469 (1943)
 4. McCay, C.M. Diet and aging. Vitam. Horm. 7, 147 (1949)
 5. Ross, M.H. and Bras, G. Tumour incidence patterns and nutrition in the rat. J. Nutr. 87, 245 (1965)
 6. Ross, M.H., Bras, G. and Ragbeer, M.S. Influence of protein and caloric intake upon spontaneous tumour incidence of the anterior pituitary gland of the rat. J. Nutr. 100, 177 (1970)
 7. Srivastava, U. Acid cathepsin activity in various organs of undernourished rats as well as their progeny. Nutr. Rep. Inter. 16, 285 (1977)
 8. Srivastava, U., Vu, M.-L., Bhargava, S. and Goswami, T. Metabolism of nucleic acids and protein in the liver, brain and kidney of female rats subjected to dietary restriction during the period of gestation as well as the period of growth, gestation and lactation. Can. J. Physiol. Pharmacol. 50, 832 (1972)
 9. Srivastava, U., Vu, M.-L. and Goswami, T. Maternal dietary deficiency and cellular development of progeny in the rat. J. Nutr. 104, 512 (1974)
 10. Srivastava, U., Goswami, T. and Vu, M.-L. Metabolism of protein and ribonucleic acid in organs of the young of undernourished female rats. I. Changes in the liver, brain and kidney. II. Changes in the pancreas, spleen and thymus. III. Changes in the lung, cardiac and skeletal muscle. Nutr. Rep. Inter. 17, 237 (1978)
 11. Srivastava, U., Ganguli, P.K., Brasseur, R. and Gyenes, L. The metabolism of liver RNA in adult rats subjected to prolonged dietary restriction during the period of growth and development. Nutr. Rep. Inter. 17, 367 (1978)
 12. Winick, M. Nutrition and cell growth. Nutr. Rev. 27, 195 (1968)
 13. Winick, M. Malnutrition and Brain Development. Oxford University Press, London-New York, 1976, p. 167

NUTRITION REPORTS INTERNATIONAL

14. Winick, M. and Noble, A. Cellular response in rats during malnutrition at various ages. *J. Nutr.* 89, 300 (1966)
 15. Winick, M., Brasel, J.A. and Rosso, P. Nutrition and cell growth. In *Nutrition and Development* (M. Winick, editor), John Wiley & Sons, New York, 1972, p. 49
 16. Winick, M. and Rosso, P. Brain DNA synthesis in protein-calorie malnutrition. In *Protein-Calorie Malnutrition* (R.E. Olson, editor), Academic Press, New York, 1975, p. 93
 17. Munro, H.N. and Fleck, A. Recent developments in the measurement of nucleic acids in biological fluids. *Analyst* 91, 77 (1966)
 18. Burton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62, 315 (1956)
 19. Goa, J. A micro-biuret method for protein determination: determination of total protein in cerebrospinal fluid. *Scand. J. Clin. Lab. Invest.* 5, 216 (1953)
 20. Dallman, P.R. Malnutrition: incorporation of thymidine ³H into nuclear and mitochondrial DNA. *J. Cell Biol.* 51, 549 (1971)
 21. Haider, M. and Tarver, H. Effect of diet on protein synthesis and nucleic acid levels in rat liver. *J. Nutr.* 99, 433 (1969)
 22. Goswami, T. and Srivastava, U. Maternal dietary deficiency and its effect on the metabolism of nucleic acids and proteins. Effect of exchanging the young, during the lactation period, between control and undernourished female rats. *Can. J. Physiol. Pharmacol.* 56, 274 (1978)
 23. Jasper, H.C. and Brasel, J.A. Rat liver DNA synthesis during the "catch-up" growth of nutritional rehabilitation. *J. Nutr.* 104, 405 (1974)
 24. Chopin, S.F. Changes in protein metabolism with age affect rehabilitation potential. *Geriatrics* 31, 52 (1976)
 25. Srivastava, U. Polyribosome concentration of mouse skeletal muscle as a function of age. *Arch. Biochem. Biophys.* 130, 129 (1969)
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LOSS OF BODY FAT IN HEALTHY WOMEN TAKING A VERY-LOW-CALORIE DIET

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ABSTRACT

We have studied the compositional changes of the body during very-low-calorie dieting in 5 healthy women (age 28-49 y; body mass index 20.7-26.6 kg.m⁻²), who wanted to lose weight for appearance's sake. The diet (The Micro Diet®, Uni-Vite 330; Uni-Vite Nutrition, Great Missenden, UK) consisted of (per day): 330 kcal, 42 g protein, 35 g carbohydrate, 3 g fat, minerals and vitamins. After 14 days of dieting body weight had decreased by 5.2 ± 1.0 kg (mean ± SD), and body fat (measured by underwater weighing) by 2.5 ± 1.8 kg. Thus 48% of the weight loss was pure fat. As it is known that "obesity tissue" involved in weight changes contains 64% fat, it can be calculated that this tissue on average accounted for 75% of the total weight loss. This implies that short-term use of the very-low-calorie diet causes selective breakdown of fat tissue while lean body mass is spared for the most part.

INTRODUCTION

It is important to know the compositional changes of the body during dieting. Reduction of energy intake may produce net loss of body protein with a negative nitrogen balance (1). Excessive protein loss may cause muscle wasting and possible impairment of immunocompetence and increased susceptibility to infection (2, 3).

The use of very-low-calorie diets containing high quality protein supplemented with minerals has been proven to be a safe and effective method to achieve weight loss (4, 5). In obese patients (mean body mass index, 37.5 kg.m⁻²) the consumption of a very-low-calorie diet (1.34 MJ/day) for 4 weeks was found to cause a body weight loss of 10 kg with only a 4% loss of calculated total body nitrogen (6). Wechsler et al. (7) reported that obese patients (body weight, 184% of ideal) on a 1-MJ/day very-low-calorie diet reached nitrogen balance within 3 weeks. Body weight fell by 12 kg in 4 weeks, and only 3.8% of the weight loss was protein (7). Thus very-low-calorie diets prevent excessive breakdown of body protein.

The above-mentioned studies have been performed with massively obese subjects. However, a great number of subjects using very-low-calorie diets are not overtly obese; the subjects only want to lose a few pounds for appearance's sake. In subjects falling into this category, we have now determined body composition during very-low-calorie dieting.

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SUBJECTS AND METHODS

Characteristics of the subjects are shown in Table 1. The women were all apparently healthy.

On day 0 of the study body weight was determined after an overnight fast, and underwater weighing was performed. Then, the subjects received a very-low-calorie diet as the sole source of nutrition for a period of 7 or 14 days. The diet (The Micro Diet®, Uni-Vite 330; Uni-Vite Nutrition, Roan House, High Street, Great Missenden, Buckinghamshire HP16 0BG, UK) consisted of (per day): 330 kcal (1.4 MJ), 42 g protein, 35 g carbohydrate, 3 g fat, 1.5 g sodium, 2.01 g potassium, 1.8 g chloride, 0.9 g calcium, 0.8 g phosphorus, 0.35 g magnesium, 0.45 g choline, 0.12 g inositol, 20 mg iron, 20 mg zinc, 2 mg copper, 2.9 mg manganese, 0.16 mg molybdenum, 0.06 mg iodine, 1 mg vitamin A, 70 mg vitamin C, 10 mg vitamin E, 2 mg thiamin, 2 mg riboflavin, 3 mg pyridoxine, 19 mg nicotinamide, 7 mg pantothenic acid, 0.4 mg folic acid, 0.2 mg biotin, 70 µg vitamin K₁, 11 µg vitamin D₃, 5 µg vitamin B₁₂, 60 µg selenium and 60 µg chromium.

The diet was provided in powder form, and it was mixed with water before use (total intake: 750 ml per day in three portions). In addition to the liquid diet, the subjects drank 1.5 to 2.5 l of water per day.

Underwater weighing was performed according to Behnke et al. (8). Volume correction was made for residual lung volume using the helium dilution technique. Body density (mass/volume) was calculated, and percentage of body fat was then calculated on the basis of dividing the body into two components: fat (density, 0.9000 g/ml at 37 °C), and fat-free mass (density, 1.1000 g/ml at 37 °C). Each experimental subject was weighed underwater in duplicate. Reproducibility of the method was assessed by weighing 4 other subjects while on their habitual diets, each day at the same time for a period of 5 consecutive days. Reproducibility of body-volume measurement (coefficient of variation) was found to be 0.38%.

RESULTS AND DISCUSSION

The diet was very well accepted by all subjects, and this held for the taste, simplicity of preparation and the lack of hunger. The subjects did not claim to experience serious side effects while dieting.

Table I shows that the women lost on average 3.2 kg after 7 days, the range of individual values being 2.1 to 4.1 kg. During the second week of dieting the rate of weight loss was reduced by about 50%. After 14 days mean weight loss was 5.2 kg.

During the first week, fat loss accounted on average for 53% of total weight loss. This value may be somewhat biased since in woman no. 4 there actually was an increase in body fat during the first week of dieting. In the other 4 women, 61 to 76% of weight loss in the first week was fat.

It is unlikely that the remarkable outcome in subject no. 4 was due to analytical error. The duplicates for body fat measurement on average

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Table 1. Effect of very-low-calorie dieting on body fat in five healthy women

	1	2	3	4	5	Mean
Age	28	45	45	49	43	42
Body mass index (kg/m ²)	20.7	25.2	26.6	24.3	23.6	24.1
Body weight (kg)						
day 0	53.1	72.8	64.0	71.2	62.7	64.8
Percentage body fat (%)						
day 0	30.9	33.3	36.9	38.4	27.8	33.5
Body fat mass (kg)						
day 0	16.4	24.2	23.6	27.3	17.4	21.8
Body weight change (kg)						
days 0- 7	-2.1	-4.1	-4.1	-3.0	-2.9	-3.2
days 7-14		-2.0	-2.0	-1.3	-1.5	-1.7
days 0-14		-6.1	-6.1	-4.3	-4.4	-5.2
Body fat change (kg)						
days 0- 7	-1.6	-2.8	-2.5	+0.3	-1.8	-1.7
days 7-14		-1.6	-0.8	-0.4	-0.5	-0.8
days 0-14		-4.4	-3.3	-0.1	-2.3	-2.5

differed only 1.9% (range 1.3 to 2.6%) from the mean value. Furthermore, the percentage of body fat was not only found increased after one week (absolute value, 40.6%), but also after two weeks (absolute value, 40.7%). Thus, it appears that this woman responded to the very-low-calorie diet in an aberrant manner.

After 14 days of dieting mean body weight had decreased by 5.2 kg, and 48% of this weight loss was fat. The total fat loss (2.5 kg) represents 22500 kcal. Thus it can be calculated that the subjects while on the diet may have consumed 1607 kcal/day less than while on their habitual diet. This is of course a rough estimate as energy expenditure decreases when body weight is lowered.

It should be noted that we have actually measured pure fat, and not fat tissue. From measurements in experiments on men gaining weight from overeating, and losing weight from semi-starvation, it appears that "obesity tissue", which is involved in weight changes, is composed of 64 per cent fat (9). Thus it can then be calculated that this fat tissue on average accounted for 75% of the total weight loss after 14 days. This implies that short-term use of the very-low-calorie diet causes selective breakdown of fat tissue, while lean body mass is spared for the most part.

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REFERENCES

1. Ball, M.F., J.J. Canary and L.H. Kyle. Comparative effects of caloric restriction and total starvation on body composition in obesity. *Ann. Intern. Med.* 67, 60 (1967).
2. Alexander, J.W. Emerging concepts in the control of surgical infections. *Surgery* 75, 934 (1974).
3. Chandra, R.K. Nutrition as a critical determinant in susceptibility to infection. *Wld Rev. Nutr. Dietetics* 25, 166 (1976).
4. Vertes, V., S.M. Genuth and I.M. Hazelton. Supplemented fasting as a large-scale outpatient program. *JAMA* 238, 2151 (1977).
5. Howard, A.N. The historical development, efficacy and safety of very-low-calorie diets. *Int. J. Obesity* 5, 195 (1981).
6. Wilson, J.H.P. and S.W.J. Lamberts. Nitrogen balance in obese patients receiving a very low calorie liquid formula diet. *Am. J. Clin. Nutr.* 32, 1612 (1979).
7. Wechsler, J.G., H.H. Ditschuneit, P. Malfertheiner and H. Ditschuneit. Stickstoffbilanzen während modifizierten Fastens. *Dtsch. Med. Wschr.* 105, 58 (1980).
8. Behnke, A.R., B.G. Feen and W.C. Welham. The specific gravity of healthy men. *JAMA* 118, 495 (1942).
9. Grande, F. and A. Keys. Body weight, body composition and calorie status. In: *Modern Nutrition in Health and Disease* (edited by R.S. Goodhart and M.E. Shils), Lea & Febiger, Philadelphia, 1980, p.3.

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EFFECT OF ORAL ZINC SUPPLEMENTATION UPON PLASMA LIPIDS, BLOOD PRESSURE, AND OTHER VARIABLES IN YOUNG ADULT WHITE MALES

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ABSTRACT

Young adult white males (N=23) were given 50 mg Zn as zinc gluconate orally for 6 wk. Subjects were instructed to eliminate food items high in copper, fiber, and phytic acid. Three-day diet records were collected biweekly and overnight urine and fasting blood samples were collected once before Zn supplementation began for baseline data and 6 wk later. A diet Zn:Cu intake ratio of 60:1 was recorded from diet records and zinc supplementation for the 6 wk experiment. As expected, plasma and urine zinc levels were greater at wk 6 compared to baseline. Diastolic pressure was decreased at the end of the experiment. Plasma cholesterol levels decreased and plasma HDL-cholesterol levels increased, but the differences were not significant. The data suggest that the effects of oral Zn supplementation in males with marginal diet Cu intake differs from results obtained with animals.

INTRODUCTION

A possible etiology of coronary heart disease is an imbalance of copper and zinc (1). Such an imbalance could result from a dietary copper deficiency, a high dietary zinc intake, or both. Rat studies have shown a hypercholesterolemic effect as a result of copper deficiency (2-4). Elevations in serum triglycerides and serum glucose have been observed in copper deficient rats (4, 5). According to the zinc copper hypothesis, excess dietary zinc should block the uptake of available copper and could result in copper deficiency when copper is present in the diet in marginal amounts.

Woo et al. (6) failed to note changes in serum cholesterol, HDL-cholesterol, or triglycerides when rats were fed high levels of

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dietary zinc. Furthermore, Fischer et al. (7) fed rats diets containing copper:zinc ratios comparable to those found in North American diets and found no changes in serum or liver total, esterified, or free cholesterol.

The relationship of copper and zinc balance in humans upon serum lipids, in particular cholesterol, is uncertain. Fischer and Collins (8) did not note any relationship between serum copper and zinc concentrations with serum HDL- or LDL-cholesterol, and triglycerides in humans. Perhaps serum levels of copper and zinc are poor indicators of the status of these two minerals (9). A previous cross-sectional study (10) on young adults suggested lower HDL-cholesterol was associated with both higher dietary zinc intakes and serum copper:zinc ratios. Hair copper levels were positively associated with higher cholesterol concentrations. Higher serum copper concentrations were associated with higher triglyceride concentrations and with lower glucose concentrations. With respect to blood pressure, higher caloric and dietary copper intakes were associated with higher systolic and diastolic pressures, whereas higher dietary zinc intakes were associated with lower pressures (11). Freeland-Graves et al. (12) demonstrated that females given 100 mg Zn/d orally had a temporary decline in HDL-cholesterol. No effect upon serum cholesterol and triglycerides were observed. Plasma cholesterol levels were negatively correlated with dietary copper intake as determined by diet records. Hooper et al. (13) has shown that pharmacological doses of zinc lowered HDL-cholesterol levels in men.

Human studies to evaluate the effects of oral zinc supplementation upon serum lipids and blood pressure are lacking. The possible deleterious effects of mineral supplements over an extended period of time is cause for concern. Since Americans generally consume a low copper diet, a copper/zinc imbalance would appear possible when consuming elevated or pharmacological doses of zinc.

The objective of the present study was to determine if oral zinc supplementation of 50 mg/d would alter serum lipids and blood pressure over a 6 wk period in white young adult males. The study was designed such that copper intake by the subjects was marginal and each subject served as their own control.

METHODS AND MATERIALS

White male subjects between the ages of 18 and 29 yr were recruited in the Fall of 1983 from the university community by classroom visitations. The protocol and experimental design were approved for human subjects by the university's Institutional Review Board. Subjects were paid \$50 for their participation. Subjects were interviewed concerning medications prescribed to them, known medical problems, including cardiovascular disorders and hypertension, family history of hypertension, smoking habits, and physical activity. Subjects selected were told to eliminate vitamin and mineral supplements that they may currently be consuming. None of the participants were taking supplements as verified by the interview.

Several restrictions were placed on participants. Only non-smokers were allowed to participate. Subjects could not be more or less than 20% deviated from their normal weight for height as determined by Metropolitan Life Insurance Tables and modified by Ross Laboratories (14). Subjects accepted for the study normally participated in no more than 3 hr/wk of jogging, tennis, or other type of planned physical activity. Initially 25 subjects were recruited to participate in the study. Twenty-three subjects completed the 6 wk study. One subject became ill with mononucleosis, and another subject was dropped from the study due to failure to adhere to protocol. All subjects included in the data analysis were free from any biochemical abnormalities as determined from a SMAC test (Columbus Pathology Laboratories, Columbus, MS). Subjects were contacted by phone weekly throughout the study to help insure adherence to protocol.

Experimental Design. Subjects were requested to consume 50 mg zinc per day as zinc gluconate with a full glass of water at the evening meal for 6 wk. Before supplementation began an overnight fasting blood sample and a morning first void urine sample were collected in plastic containers from each subject for baseline data. Subjects were also requested to complete a 3-d diet record. After supplementation began, blood and urine were collected 6 wk thereafter. Three-d diet records were collected biweekly. To lower both the amount of copper and fiber in the diet, subjects were requested to limit the intake of certain food items. The diet was also moderate in phytic acid content and calcium, to prevent interference in zinc absorption (diet available from D.M.M.). Subjects were requested to maintain a constant caloric intake during the study. Diet records were used to confirm this. Height in cm, weight in kg, triceps thickness, and blood pressure were also recorded at the beginning of the study and at the completion.

Diet Records. Instructions were given to subjects on how to keep a 3-d diet record. Food items consumed on a Tuesday, Wednesday, and Thursday of each two week period were recorded. Sheets were developed for subjects to record food items eaten, how they were prepared, and the total amounts eaten. A measuring cup, utensils, and portion models were supplied to quantify accurately food consumed. A diet interviewer reviewed the completed record with each subject for accuracy and completeness. From the record sheets, daily intakes of calories, protein, carbohydrate, fat, and crude fiber values were calculated from U.S.D.A. Agricultural Handbook No. 456. Daily intakes of copper and zinc were calculated from the tables of U.S.D.A. Handbook No. 8-1, No. 8-5, Freeland and Cousins (15), Murphy et al. (16), and Pennington and Calloway (17).

Blood Pressure Measurements. Blood pressure was recorded from subjects in the sitting position with an electronic sphygmomanometer (Dyna-Med, Inc., Carlsbad, CA). Three determinations were made and averaged to develop a mean individual value. Systolic pressure was defined as the first of consecutive Korotoff sounds and diastolic as the disappearance of the sounds. Data was expressed in mm Hg.

Urine Analysis. Subjects submitted an overnight urine sample on

the day on which their blood was collected. Samples were collected in plastic containers and the urine volumes were recorded in the lab. Five-milliliters of HCl per 100 ml of urine were added as a preservative. Urine samples were frozen until analyzed.

When urine samples were thawed, creatinine was determined by the Jaffe reaction. Urine copper was determined directly by flame atomic absorption spectrophotometry (AAS). For zinc analysis, urine samples were diluted with deionized-distilled water and analyzed by flame AAS. Data were expressed as mg of copper or zinc per g of creatinine excreted.

Blood Collection. An overnight 12 h fasting blood sample was collected by a medical technician by venal puncture. Heparinized Vacutainer tubes prepared for trace element analysis were used for blood collections (Fisher Scientific Co., Fairlawn, N.J.). Some of the blood collected was used for hemoglobin and hematocrit determinations. The remaining blood sample was centrifuged at 400 X g for 20 min to obtain plasma.

Hemoglobin and Hematocrit Determination. Hemoglobin values were determined spectrophotometrically as cyanmethemoglobin in a Fisher Hemophotometer using Drabkin's reagent. The data was expressed as g of hemoglobin per 100 ml of blood. Hematocrits were determined by centrifuging blood filled capillary tubes in a micro-hematocrit centrifuge and reading the values in percent on a micro-capillary tube reader.

Plasma Copper and Zinc Analysis. After centrifugation of the blood, an aliquot of the plasma was removed and frozen until analysis of copper and zinc. Samples were thawed and diluted 1:5 with deionized-distilled water for copper and zinc analysis. Copper and zinc levels were determined by flame AAS and expressed as ug/ml or parts per million (ppm).

Plasma Lipids and Glucose. Plasma cholesterol, HDL-cholesterol, triglycerides and glucose levels were determined on the same day as when the blood samples were collected.

Total cholesterol and HDL-cholesterol were assayed with a Stanbio Kit (Stanbio Laboratory, Inc., San Antonio, TX). Total plasma cholesterol determination was based on the Leibermann-Burchard reaction. High-density-lipoprotein cholesterol was determined on another plasma aliquot by precipitation with a 1 M solution of $MgCl_2$ in 1 % aqueous Dextran sulfate to remove LDL- and VLDL- cholesterol. The supernatant was assayed for cholesterol based on the Leibermann-Burchard reaction. The absorbance of all specimens and standards were recorded at 625 nm within 30 min using a Bausch and Lomb 88 spectrophotometer.

Plasma triglycerides were assayed with a Stanbio Reagent kit using an enzymatic procedure. In this method a microbial lipoprotein lipase hydrolyzes the serum triglycerides to glycerol and free fatty

acids. The glycerol is measured by means of a series of enzymatic reactions until NADH, which is produced, reduces the dye iodonitrotetrazolium to a formazan (an intensely colored dye). The formazan dye produced is proportional to the initial glycerol concentration and the absorbance is recorded at 500 nm on a Bausch and Lomb 88 within 2 hr.

Plasma glucose was assayed on a YSI Model 23A glucose analyzer (Yellow Spring International Co., Inc., Yellow Springs, Ohio). Reference serum, purchased from Sigma Chemical Co. (St. Louis, MO) was used to insure the reliability of the triglyceride and cholesterol assays. All lipid and glucose concentrations were expressed as mg/100 ml plasma.

To check our procedures, on one occasion blood samples were sent to an outside laboratory for analysis. For plasma total cholesterol, triglycerides, and glucose, correlation coefficients of 0.90, 0.93, 0.91 ($P \leq 0.001$) were calculated, respectively, between our values and the outside laboratory.

AAS Analytical Procedure. A Perkin-Elmer flame AAS (Model 2380, Perkin-Elmer Corp., Norwalk, CT) was used to analyze plasma and urine for copper and zinc. Procedures and instrument settings used were as suggested by the manufacturer (18). Urine collection containers and glassware used were rinsed in 3 N HNO₃ followed by deionized-distilled water to minimize trace element contamination. Standards were prepared fresh daily from Fisher Certified Atomic Absorption Standards (Fisher Scientific Co., Fairlawn, N.J.).

Statistical Analysis. Dietary data for each biweekly collection period were analyzed by ANOVA to determine if differences existed over time for intakes of calories, protein, carbohydrate, fat, protein, copper, zinc, and fiber. Mean values for blood chemistries, anthropometrics, blood pressures, and dietary values were computed and differences between baseline and wk 6 were determined by paired t-test (19).

RESULTS

Twenty-three subjects completed the 6 wk study. Intakes of calories, fat, protein, carbohydrate, fiber, zinc, and copper did not differ by week (Table I, $P > 0.05$). Age, weight:height index, and triceps skinfold thickness are also present in Table I.

Diastolic pressure showed a significant decrease (Table II, $P \leq 0.01$) at wk 6 compared to baseline. Plasma and urine zinc increased at wk 6 ($P \leq 0.01$). Plasma cholesterol level tended to decrease and plasma HDL-cholesterol values tended to increase at wk 6 but the differences were not significant ($P > 0.05$).

DISCUSSION

The data suggest that zinc supplementation had no significant effect upon mean plasma lipid levels. Diastolic pressure did appear to

Table I. Mean Age, Anthropometric, and Dietary Intakes of Subjects (\pm S.E.)

Variable	WEEK					P
	Baseline	2	4	6	S.E.	
Age (yr) ¹	21.1				± 2.10	
Anthropometric ¹						
Weight:Height	0.44				± 0.04	
Triceps Skinfold Thickness (mm)	13.35				± 3.41	N.S. ²
Protein (g/d)	109.7	110.7	99.9	116.0	± 6.63	N.S.
Fat (g/d)	129.8	131.3	132.1	135.2	± 9.49	N.S.
Carbohydrate (g/d)	284.5	297.7	283.5	282.7	± 19.08	N.S.
Fiber (g/d)	3.79	3.82	3.84	3.21	± 0.359	N.S.
Zinc (mg/d) ³	11.4	11.9	11.6	12.4	± 0.59	N.S.
Copper (mg/d)	0.95	0.93	1.00	1.01	± 0.062	N.S.

¹Overall 6 wk mean.

²N.S. denotes no statistical significance ($P > 0.05$).

³Values do not include supplementation with 50 mg Zn/d.

be a variable that decreased in response to zinc supplementation.

Based on several animal studies (2, 20-22) and on Klevay's hypothesis (1), the relationship for zinc dose with plasma cholesterol levels appear inconsistent. High diet zinc levels could decrease copper absorption and lead to elevated plasma cholesterol levels according to animal data. Our data indicated a mean daily intake of 0.97 mg for copper. The mean daily intake of zinc from food sources was 11.81 mg. The total daily intake of zinc would be slightly above 60 mg. This would give a diet zinc to copper ratio of 60:1. Most animal studies have shown changes in serum cholesterol when the ratio was 40:1. Koo and Williams (23) studied zinc deficiency and serum lipoprotein-cholesterol metabolism in rats. Zinc deficiency produced a decrease in total cholesterol, primarily in the HDL fraction. Sandstead et al. (24) observed a 22% decline in serum total cholesterol in a young male on a marginal zinc diet.

Table II. Mean Values for Blood Chemistries, Blood Pressure, and Plasma and Urine Copper and Zinc Concentrations at Baseline and Week 6 (\pm S.E.)

Variable	Baseline	Week 6	P
Plasma			
Cholesterol (mg/100ml)	156.8 \pm 7.28	149.5 \pm 6.70	N.S. ¹
Plasma HDL-cholesterol (mg/100ml)	60.3 \pm 2.05	69.9 \pm 2.97	N.S.
Plasma Triglycerides (mg/100ml)	87.0 \pm 7.26	86.0 \pm 6.31	N.S.
Systolic Pressure (mmHg)	121.1 \pm 2.15	119.4 \pm 2.23	N.S.
Diastolic Pressure (mmHg)	74.0 \pm 1.74	61.5 \pm 2.18	0.01
Plasma Zn (ppm)	0.76 \pm .027	1.05 \pm .038	0.01
Plasma Cu (ppm)	1.08 \pm .02	1.12 \pm .033	N.S.
Urine Zn (mg/g creatinine)	0.40 \pm .	0.64 \pm .058	0.01
Urine Cu (mg/g creatinine)	0.29 \pm .054	0.36 \pm .023	N.S.
Hematocrit (%)	46.8 \pm .53	46.1 \pm .72	N.S.
Hemoglobin (g/100ml)	16.1 \pm .20	15.2 \pm .20	N.S.

¹N.S. denotes no statistical significance ($P > 0.05$).

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A previous study on human subjects demonstrated a negative association for diet zinc with serum cholesterol (10). Hooper et al (13) noted decreases in HDL-cholesterol when males were given pharmacological doses of zinc. Freeland-Graves (12) noted only a transient decline in serum HDL-cholesterol levels. Total serum cholesterol levels did not appear to be effected by zinc dose levels in their studies. Medeiros et al. (10) observed a negative relationship for diet zinc with serum HDL-cholesterol levels. In the present study, zinc dose did not appear to affect plasma HDL-cholesterol levels.

Zinc supplementation did not decrease plasma and urine copper levels as one may expect since the two minerals are antagonist for some biological functions.

Zinc dose did not appear to have any effect upon plasma glucose. One might expect a change in plasma glucose levels via possible changes in copper status. An inverse relationship between copper and serum glucose levels (10) and decreased glucose tolerance in copper deficient animals (5) have been reported.

Our results suggest that supplementation with 50 mg Zn/d coupled with marginal diet copper intake was associated with lower diastolic blood pressure and elevated plasma and urine zinc levels. Plasma lipids were not affected by daily zinc supplementation at the current dose used. A study utilizing two zinc doses (50 and 75 mg Zn/d) and a placebo for 12 wk is underway.

REFERENCES

1. Klevay, L.M. Coronary heart disease: the zinc/copper hypothesis. *Am. J. Clin. Nutr.* 28, 764 (1975).
2. Klevay, L.M. Hypercholesterolemia in rats produced by an increase in the ratio of zinc to copper ingested. *Am. J. Clin. Nutr.* 26, 1060 (1973).
3. Klevay, L.M. Interactions of copper and zinc in cardiovascular disease. *Ann. NY. Acad. Sci.* 355, 140 (1983).
4. Wu, B.N., Medeiros, D.M., Lin, K.N., Thorne, B.M. Long term effects of dietary copper and sodium upon blood pressure in the Long-Evans rat. *Nutr. Res.* 4, 305 (1984).
5. Hassel, C.A., Marchello, J.A., Lei, K.Y. Impaired glucose tolerance in copper deficient rats. *J. Nutr.* 113, 1081 (1983).
6. Woo, W., Gibbs, D., Hooper, P.L. Garry, P.J. Zinc and lipid metabolism. *Am. J. Clin. Nutr.* 34, 120 (1981).
7. Fischer, P.W., Giroux, A., Belonje, B., Shah, B. The effect of dietary copper and zinc in cholesterol metabolism. *Am. J. Clin. Nutr.* 33, 1019 (1980).

NUTRITION REPORTS INTERNATIONAL

8. Fischer, P.W., Collins, M.W. Relationship between serum zinc and copper and risk factors associated with cardiovascular disease. *Am. J. Clin. Nutr.* 34, 595 (1983).
9. Klevay L.M. Reply to letter by Fischer and Collins. *Am. J. Clin. Nutr.* 34, 597 (1981).
10. Medeiros, D.M., Pellum, L., Brown, B. Serum lipids and glucose as associated with hemoglobin levels and copper and zinc intake in young adults. *Life Sci.* 32, 1897 (1983).
11. Medeiros, D.M., Brown, B.J. Blood pressure in young adults as influenced by copper and zinc intake. *Biol. Trace Element Res.* 5, 165 (1983).
12. Freeland-Graves, J.H., Friedman, B.J., Han, W.H., Shorey, R.A.L., Young, R. Effect of zinc supplementation on plasma high-density-lipoprotein cholesterol and zinc. *Am. J. Clin. Nutr.* 35, 988 (1982).
13. Hooper, P.L., Visionti, L., Gray, P.J., Johnson, G.E. Zinc lowers high-density lipoprotein-cholesterol levels. *JAMA* 244, 1960 (1980).
14. Assessing nutritional status. Ross Laboratories, Columbus, OH (1978).
15. Freeland, J.H., Cousins, R.J. Zinc content of selected foods. *J. Am. Dietet Assoc.* 68, 526 (1976).
16. Murphy, E.W., Willis, B.W., Watt, B.K. Provisional tables on the zinc content of foods. *J. Am. Dietet. Assoc.* 66, 345 (1975).
17. Pennington, J.T., Calloway, D.H. Copper content of foods. *J. Am. Dietet. Assoc.* 63, 143 (1973).
18. Perkin-Elmer Corporation. Analytical methods for atomic absorption spectrophotometry, Norwalk, CT, (1976).
19. Steel, R.D.G., Torrie, J.H. Principles and procedures of statistics: a biometrical approach. New York: McGraw Hill Book Co, (1980).
20. Allen, K.G.D., Klevay, L.M. Cholesterolemia and cardiovascular abnormalities in rats caused by copper deficiency. *Atherosclerosis* 29, 81 (1978).
21. Allen, K.G.D., Klevay, L.M. Hyperlipoproteinemia in rats due to copper deficiency. *Nutr. Rep. Int.* 22, 295 (1980).
22. Lei, K.Y. Cholesterol metabolism in copper deficient rats. *Nutr. Rep. Int.*, 15, 597 (1977).

NUTRITION REPORTS INTERNATIONAL

23. Koo, S.I., Williams, D.A. Relationship between the nutritional status of zinc and cholesterol concentration of serum lipoproteins in adult male rats. *Am. J. Clin. Nutr.* 34, 2376 (1981).
24. Sandstead, H., Klevay, L., Mahalko, J., Inman, L., Bolonchuk, W., Lukaski, H., Lykken, G., Kramer, T., Johnson, L., Milne, D., Wallwork, J. Marginal Zn nutriture: effects on lipid metabolism and plasma zinc. *Am. J. Clin. Nutr.* 33, 944 (Abstr) (1980).

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EFFECT OF CELLULOSE INCORPORATION IN A LOW FIBER DIET ON FECAL EXCRETION AND DIGESTIBILITY OF NUTRIENTS IN ADOLESCENT GIRLS

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ABSTRACT

Balance studies on nine adolescent girls were conducted in two trials of three weeks each on low and high fiber diets. The high fiber diet contained 21 g pure cellulose added to the low fiber diet. Food and fecal samples were collected and analysed for nitrogen, energy, fat and ash. The diet and nutrient intake of the subjects was almost the same during both trials except for fiber. Addition of cellulose to low fiber diet significantly ($P \leq 0.01$) decreased the body weight. Apparent digestibilities of nitrogen, energy, fat and ash were significantly ($P \leq 0.01$) decreased on high fiber diet. Therefore, high cellulose intakes may not be beneficial.

INTRODUCTION

Several types of dietary fiber may be important in human nutrition and it is now clear that these do not have the same biological effect. Less attention has been given to the individual components of dietary fiber such as cellulose, hemicellulose, pectin and lignin (1). Dietary fiber has potential for binding fat, nitrogen, cholesterol, minerals etc. The excessive intake of fiber may result in loss of important nutrients (2). Energy, fat, nitrogen and mineral absorption appear to be markedly decreased by increased fiber intake (3).

Various parameters were measured in adolescent girls consuming a low fiber diet and a diet high in fiber from cellulose. When the subjects consumed the high fiber diet, number of defecations and wet and dry weight of stools increased (4). Fecal excretions of calcium, phosphorus and iron were greater on the high fiber diet than on the low fiber diet (5). Few data are available on the effect of feeding fiber from non-cereal sources on nutrient absorption. The present study was undertaken to examine the effect of cellulose on digestibility of nutrients.

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MATERIAL AND METHODS

The study was conducted on nine healthy adolescent girls of 16 to 18 years of age with an average weight of 48 kg and average height of 155 cm. The balance study was divided into two parts. During the first part of the study, the subjects consumed a low fiber diet based on refined cereals, eggs, milk and milk products, potatoes and low fiber vegetables. For the second part of the study, the subjects were fed the same diet with the addition of 21 g nutritional grade cellulose per day in snacks and sweet dishes for 21 days. The advantage of using cellulose was that the fiber content of the diet could be increased without altering the composition of the diet consumed.

For each diet, the first 2 wk were taken as an adjustment period, after which food and fecal samples were collected for 1 week. The designations "low fiber diet" and "high fiber diet" refer only to the relative fiber content of the two diets fed in this study. During the experimental period weighed amounts of egg, butter, milk, soup, fruit and salad were served to all the subjects. Other food items such as bread, rice and potatoes were allowed ad libitum.

The daily food intake of each subject was recorded in a proforma. All items of food consumed by each subject in each meal were weighed and measured. One fifth aliquots of each food consumed by the subjects in all the meals were accurately weighed and measured and kept in separate plastic containers. The 7-day samples were homogenized and analysed for proximate composition, cellulose and gross energy.

Charcoal tablets were used as a marker for collection of fecal samples which were collected and weighed for 7 days, homogenized and analysed for proximate composition and gross energy.

The proximate composition of the food and fecal samples was determined using the AOAC method (6), and gross energy by the Toshniwal Bomb Calorimeter. The crude protein was calculated by using the conversion factor of $N \times 6.25$, soluble carbohydrates were calculated by the difference method. Weende crude fiber of food samples were estimated by the AOAC method (6) and cellulose by the method of Crampton and Maynard(7).

The apparent digestibility of nutrients was calculated by taking the difference between intake and fecal excretion and expressed as percentage of intake. Data were analysed statistically by using 't' test.

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RESULTS

The data on the diet and nutrient intake of the subjects during low and high fiber diets are shown in Table I.

TABLE I

Average daily food and nutrient intake of nine adolescent girls on low and high fiber diets (dry matter basis).

	Diet and nutrient intake		Diet composition (%)	
	Low fiber diet	High fiber diet	Low fiber diet	High fiber diet
Food intake (g)	473 \pm 7.2 ¹	476 \pm 5.9	-	-
Crude protein (g)	71.1 \pm 0.65	70.9 \pm 0.57	15.1 \pm 0.14	14.9 \pm 0.12
Crude fat (g)	77.9 \pm 1.12	78.4 \pm 0.55	16.5 \pm 0.22	16.5 \pm 0.19
Soluble carbohydrates (g)	301 \pm 5.7	291 \pm 4.7	63.5 \pm 0.30	61.2 \pm 0.30
Weende crude fiber(g)	2.6 \pm 0.07	15.3 \pm 0.06	0.6 \pm 0.01	3.2 \pm 0.04
Cellulose (g)	2.8 \pm 0.03	23.5 \pm 0.01	0.6 \pm 0.01	5.0 \pm 0.04
Gross energy (Kcal)	2294 \pm 19.9	2286 \pm 19.8	485 \pm 5.0	480 \pm 4.0
Total mineral matter(g)	20.5 \pm 0.45	20.5 \pm 0.44	4.3 \pm 0.04	4.3 \pm 0.05

¹Mean values \pm SE (n=9)

The diet and nutrient intake was almost the same on both diets except for fiber and cellulose. Cellulose was well tolerated by all the subjects. The mean body weight of the subjects on low and high fiber diets was 50.5 \pm 1.96 and 49.2 \pm 2.01 kg, respectively. There was a significant ($P \leq 0.01$) decrease in body weight on high fiber diet.

The data on intake, fecal loss and apparent digestibility of nitrogen, energy, fat and ash of the subjects fed low and high fiber diets are shown in Table II.

It is evident from Table II that the differences in the nitrogen, energy, fat and ash intakes during both trials were not significant. There was a significant ($P \leq 0.01$) increase in fecal excretion of nitrogen, energy, fat and ash on the high fiber diet. Addition of cellulose to the low

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TABLE II

Intake, fecal excretion and apparent digestibility of nitrogen, energy, fat and ash from low and high fiber diets in nine adolescent girls

	Low fiber diet	High fiber diet	Level of significance
Nitrogen			
Intake(g/day)	11.38 \pm 0.10 ¹	11.34 \pm 0.09	NS
Fecal excretion (g/day)	1.05 \pm 0.07	2.09 \pm 0.07	P \leq 0.01
Apparent digestibility(%)	90.77 \pm 0.64	81.57 \pm 0.61	P \leq 0.01
Energy			
Intake(Kcal/day)	2294 \pm 19.9	2286 \pm 19.8	NS
Fecal excretion (Kcal/day)	97 \pm 4.4	177 \pm 6.3	P \leq 0.01
Apparent digestibility(%)	94.37 \pm 0.18	92.24 \pm 0.26	P \leq 0.01
Fat			
Intake (g/day)	77.87 \pm 1.12	78.36 \pm 0.55	NS
Fecal excretion (g/day)	1.55 \pm 0.09	3.14 \pm 0.15	P \leq 0.01
Apparent digestibility (%)	98.03 \pm 0.12	95.94 \pm 0.18	P \leq 0.01
Ash			
Intake (g/day)	20.54 \pm 0.45	20.46 \pm 0.45	NS
Fecal excretion (g/day)	3.23 \pm 0.12	5.41 \pm 0.12	P \leq 0.01
Apparent digestibility (%)	84.25 \pm 0.63	73.62 \pm 0.76	P \leq 0.01

¹Mean values \pm SE (n=9)

NS - Not significant

fiber diet decreased the apparent digestibilities of these nutrients significantly (P \leq 0.01)

DISCUSSION

The results of the present investigation reveal that the high fiber diet resulted in decreased body weight. It is possible that fiber binds the nutrients and increases their excretion thus affecting the body weight adversely. Mickelson *et al.* (8) also reported decrease in body weight on high fiber bread diet in college male students.

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The high fiber diet resulted in increased fecal excretion of nitrogen, energy, fat and ash. The apparent digestibility of these nutrients was significantly ($P \leq 0.01$) decreased on high fiber diet. The dietary nutrients are more fully digested and absorbed on the low fiber diet as compared to high fiber diet. This can be due to longer stay of foods in the intestine on the low fiber diet. Beresteyn et al. (9) observed that cellulose and bran both increased the fecal fat excretion in male obese rats.

The results of the present investigation agree with those of Southgate and Durnin (10) who reported increased losses of energy, fat, nitrogen and ash in the feces on the high fiber diet as compared to those of low fiber diet. However, the diets used by Southgate and Durnin contained whole meal bread in addition to fruits and vegetables. Walker (11) also reported increased excretion of fat and nitrogen on high fiber diet based on fruits in 26 Negro Children of 9 to 12 years age.

It is generally believed that fiber decreases energy availability by hastening transport through the gut and hence increases the nitrogen and fat in the feces. Mechanisms for the decreased digestibility of fat and nitrogen associated with ingestion of some fiber source are vague. The additional fecal fat could represent bacterial lipids or a decreased absorption of dietary fat (12). Most of the evidence available supports the idea that fiber causes an increase in unabsorbed dietary fat.

In the USA and United Kingdom, the increased losses of energy, nitrogen, fat and minerals are of little consequence but might be important in developing countries like India and Pakistan in which diet intakes are low and fiber intakes are high. The depression of apparent digestibility of nitrogen may be of particular concern to those who are attempting to reduce body weight by cutting down food intake and including large amounts of fiber in the diet. Care should be taken to maintain adequate protein intake (3)

The minimum amount of fiber necessary for proper elimination and positive well being remains to be determined. It is probably less than the amount fed in this study. However, for transferring the present results into recommendation for clinical application, more research needs to be done.

REFERENCES

1. Prather, E.S. Effect of cellulose on serum lipids in

NUTRITION REPORTS INTERNATIONAL

- young women. J. Am. Diet. Assoc. 45: 230, 1964.
2. Van Soest, P.J. Fiber in the diet. Ind. J. Nutr. Diet. 13: 395, 1976.
 3. Kelsay, J.L., Behall, K.M., and Prather, E.S. Effect of fiber from fruit and vegetables on metabolic responses of human subjects. Bowel transit time, number of defecation, fecal weight, urinary excretion of energy, nitrogen and fat. Am. J. Clin. Nutr. 31: 1149, 1978.
 4. Bhat, C.M., Godara, R.B., and Kaur, A.P. Effect of cellulose supplementation on bowel behaviour, blood glucose and serum protein levels. Ind. J. Nutr. Diet. 18: 171, 1981.
 5. Godara, R.B., Kaur, A.P. and Bhat, C.M. Effect of cellulose incorporation in a low fiber diet on fecal excretion and serum levels of calcium, phosphorus and iron in adolescent girls. Am. J. Clin. Nutr. 34: 1083, 1981.
 6. AOAC. Official Methods of Analysis of the Association of Analytical Chemists, Washington DC, 1975.
 7. Crampton, E.W. and Maynard, L.M. The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. 15: 383, 1938.
 8. Mickelson, O., Makdani, D.D., Cotton, R.H., Titcomb, S.T., Colmey, J.C. and Gatty, R. Effects of a high fiber bread diet on weight loss in college age males. Am. J. Clin. Nutr. 32: 1703, 1979.
 9. Beresteyn, E.C.H., Schnaik, M. and Mogot, M.F.K. Effect of bran and cellulose on lipid metabolism in obese female zucker rats. J. Nutr. 109: 2085, 1979.
 10. Southgate, D.A.T., and Durnin, J.V.G.A. Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. Brit. J. Nutr. 24: 517, 1970.
 11. Walker, A.R.P. Effect of high crude fiber intake on transit time and absorption of nutrients in South African Negro School Children. Am. J. Clin. Nutr. 28: 1161, 1975.
 12. Slavin, J.L. and Marlett, J.A. The effect of purified cellulose on human bowel function. Fed. Proc. 37: 756, 1978.

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SOME PHYSICOCHEMICAL PROPERTIES AND COMPOSITION OF ADIPOSE TISSUE OF GOATS FED WITH DIFFERENT DIETS

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ABSTRACT

Four lots of goats fed each one with alfalfa hay, high fat, high protein and high carbohydrate supplemented diets during two months, were used.

The concentration of oleic acid was the highest of all fatty acids in subcutaneous adipose tissue. In this tissue the amount of unsaturated fatty acids was the highest for the animals fed with carbohydrate supplemented diet and the concentration of saturated fatty acids was the highest for animals fed with high fat supplemented diet.

Melting point, iodine index and refractive index were determined in subcutaneous, omental and perirenal adipose tissues. In omental the iodine index was higher than in the others. The highest value of this index was observed with carbohydrate supplemented diet.

The melting point was the least in subcutaneous adipose tissue with carbohydrate supplemented diet.

The refractive index was the least in subcutaneous adipose tissue of goat fed with alfalfa hay.

INTRODUCTION

The less consumption of saturated fat in the diet is one of the accepted factors that having positive influence in the prevention of cardiovascular diseases. For this reason, in the market there is more demand of meat of animals that have less fat content.

Different studies have showed that the fatty acid composition of bovine fat is modified by the diet (1), (2). In some cases there is variation of some physicochemical properties (3) associated to different diets.

Some authors (4) suggest that the poly unsaturated acids are responsible of the flavor of meat as a result of the

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formation of volatile compounds, as unsaturated aldehydes.

The aim of this study was to examine the fatty acid composition in subcutaneous adipose tissue and to determine some physicochemical properties of adipose tissue of goats fed with different supplemented diets.

We have not found previous reports about this specific problem in goats. In our experiment we have used goats, because it is a typical animal in Cuyo (Argentina), which meat is largely consumed by the rural population.

METHODS

Male goats, "Criolla Argentina" breed two months old, were used in this experiment. Sixteen goats were separated during two months in four lots, each one received different diets. 1: control lot: alfalfa hay and the other lots received supplemented diets with: 2: fat, 3: protein and 4: carbohydrate. The composition of the diets and the supplement of minerals and vitamins added to the water are consigned in the table N° I.

The chemical composition of the diet was determined according to the methods of AOAC (5). The animals consumed about three kilograms of it daily.

The animals were slaughtered. Immediately the total subcutaneous, omental and perirenal adipose tissues were excised. They were weighed and washed with saline solution, NaCl 0.15 M.

In order to study the effect of the different dietary treatments on the fatty acids composition of the total lipids, one gram of the subcutaneous adipose tissue was extracted according to Folch method (6). The lipid extract was submitted to saponification and methylation for preparing the methyl ester of total lipids (7). All operations were carried out under N₂ atmosphere.

A Packard, model 5840 A, gas chromatograph (Hewlett Packard Instrument Co. Inc. Chicago, Il.) equipped with an stainless steel injection splitter and a flame ionization detector was used to separate the methyl esters on a glass column of 1.80 m. containing 10% SP 2330, 100/120 chromosorb WAW. The oven temperature was programmed from 140-220°C. The injector and detector temperatures were 205°C and 260°C respectively. The N₂ flow rate was 20 ml/min. Identification of methyl esters of fatty acids was accomplished by comparing relative retention times with authentic standard, (Sigma Chem.Co).

Five grams of each adipose tissues were used to deter-

TABLE N° I

COMPOSITION OF THE DIETS AND MIX OF MINERALS AND VITAMINS ADDED TO WATER

Composition of the diets	Control	High fat	High protein hydrate	High carbo-	Minerals and Vitamins (g%)	Minerals and Vitamins (%)
Alfalfa hay*	1000	650	100	100	Cupric sulfate	5.00
Sunflower seeds*		300			Ferric sulfate	9.00
Sunflower pellet*		50	800	100	Magnesium sulfate	10.00
Maize grain*			100	800	Manganese chloride	1.50
Dry matter (%DM/kg diet)	98.10	90.65	91.01	87.16	Cobalt sulfate	1.00
Crude fiber (%DM)	32.36	28.58	20.11	6.37	Zinc sulfate	1.00
Ether extract (%DM, 105°C)	1.00	9.95	4.13	5.92	Calcium chloride	14.00
Crude protein (%DM)	20.96	21.97	32.78	18.82	Potassium iodine	0.15
Energetic value (Mcal./kg DM)	2.10	2.29	2.45	3.07	Potassium chloride	2.50
					Sodium phosphate	22.00
					Sodium chloride	31.73
					Calcium pantothenate	0.34**
					Riboflavin	1.20**
					Niacin	0.56**
					Vitamin B ₁₂	0.18***
					Vitamin A	2200.00****
					Vitamin D ₃	220.00****

The ingredients were ground- DM: Dry matter *: g/kg diet.

Water contained 2g/l of this mix of minerals and vitamins, (Commercial mix for ruminants). ** : gram, *** : micro gram, **** : International units.

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mine: refractive index, melting point and iodine index, according to the methods of AOAC, (5).

The analysis of variance was applied in order to determine the stadistic significance of the results (8).

RESULTS

The body weight and adipose tissue weight are consigned in the table II. The body weight, omental and perirenal adipose tissue weights, were not modified with the diets. The subcutaneous adipose tissue weight was the highest for animals fed with carbohydrate diet, followed for the values obtained with protein, fat and control diets.

TABLE N°II

BODY WEIGHT AND ADIPOSE TISSUE WEIGHT OF GOATS FED WITH DIFFERENT DIETS

Weight	Diets			
	A	B	C	D
Body (Kg)				
Initial	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00
Final	12.50±0.50	12.00±1.00	12.00±1.15	12.50±0.50
Adipose tissue				
(g)				
Subcutaneous	15.00±0.00	18.00±1.00	20.00±2.00	26.00±1.00
Perirenal	88.00±3.00	95.00±7.00	86.00±8.00	89.00±3.00
Omental	86.00±5.00	83.00±2.00	79.00±7.00	84.00±6.00

a-b $P < 0.05$, a-c $P < 0.01$, a-d $P < 0.001$

A:control, B:high fat, C:high protein, D:high carbohydrate.

The values are:mean \pm standard deviation from 4 animals in each case.

In subcutaneous adipose tissue the fatty acid composition was dependent of the diet. With carbohydrate diet, the percentage of unsaturated acid was higher than that with the other diets. $P < 0.001$. The major difference observed was the determined in relation to the value obtained for the fat diet. The percentage of total saturated fatty acids evaluated for the fat diet was the highest compared with the others. $P < 0.001$. Table III.

Independently of the diet, the concentration of 18:1 was the major in relation to the other saturated and unsaturated fatty acids. The 16:0 was the major of the saturated acids followed by 18:0.

The concentration of 18:2 ω 6 was the same for the different diets. The amount of 20:1 ω 9 was the least with carbohydrate diet and for the other diets there were not diffe-

TABLE N°III

PERCENTAGES OF FATTY ACID IN SUBCUTANEOUS ADIPOSE TISSUE
OF GOATS FED WITH DIFFERENT DIETS

Fatty acids	Diets			
	A	B	C	D
saturated				
C* 14:0	5.51	5.35	4.24	n.d.
C 16:0	21.95	22.66	19.66	17.05
C 18:0	11.48	14.32	13.89	13.92
Fatty acids				
unsaturated				
C 16:1	17.50	16.20	20.82	22.34
C 18:1	38.08	38.62	35.18	42.97
C 18:2 ω 6	3.04	3.17	3.31	2.99
C 20:1 ω 9	1.09	1.10	0.93	0.44
C 20:4 ω 6	1.29	0.84	2.25	0.27
Total satu- rated	38.94	42.43	37.46	30.97
Total unsa- turated	61.00	59.93	62.49	69.01

A:control, B:high fat, C:high protein D:high carbohydrate.
C*:Carbon number, : number of bonds, ω :double bond position
from methyl end.

The values are the means from 4 animals in each case.
n.d.: no detected

rences in the concentration of these fatty acids.

In relation to the physicochemical properties of the different adipose tissues, it was found that they were modified by the diets. Table IV.

Comparing the tissues it was observed that the iodine index, for animals fed with alfalfa hay was higher in the case of omental adipose tissue. For this diet, the values of this index did not show significative differences between perirenal and subcutaneous adipose tissues.

With fat diet, the iodine index was higher in omental adipose tissue, $P < 0.001$, being the least value in subcutaneous adipose tissue. $P < 0.01$. With protein diet, the value of this index was the least in subcutaneous and the highest value was observed in omental adipose tissue.

With carbohydrate diet, this index was lesser in subcutaneous adipose tissue than in omental and perirenal. Between these last tissues there were not differences.

In omental and perirenal adipose tissues, the iodine index were the least with alfalfa hay and the highest with car-

TABLE N°IV
SOME PHYSICO-CHEMICAL PARAMETERS OF ADIPOSE TISSUE OF GOATS FED WITH DIFFERENT DIETS

Diets	Adipose tissue									
	Omental		Perirenal		Subcutaneous		P <			
	M	SD	M	SD	M	SD	O-P	O-S	P-S	
Alfalfa hay										
A	a37.45	2.76	a30.05	2.66	a32.52	0.45	0.01	0.05	n.s	
B	50.35	0.03	55.96	1.05	e51.20	0.67	0.01	n.s	0.01	
C	i1.4481.00	4.04	i1.4480.00	0.82	i1.3934.00	0.25	n.s	0.01	0.01	
High fat										
A	b42.07	0.04	b38.87	2.86	b30.32	1.28	0.05	0.001	0.01	
B	51.57	2.96	53.70	3.45	f40.37	0.18	n.s	0.001	0.001	
C	j1.4495.00	5.32	j1.4501.00	3.05	j1.4534.00	0.25	n.s	0.001	0.05	
High protein										
A	c40.85	2.44	c35.50	2.85	c33.44	1.27	0.05	0.001	n.s	
B	50.62	2.07	53.27	1.94	g41.80	2.12	n.s	0.001	0.001	
C	k1.4489.00	1.92	k1.4485.00	0.50	k1.4558.00	2.50	n.s	0.01	0.01	
High carbo- hydrate										
A	d43.50	0.00	d41.83	0.74	d36.50	1.60	n.s	0.001	0.001	
B	49.17	3.20	52.85	2.45	h39.10	0.16	n.s	0.001	0.001	
C	l1.4488.00	2.65	l1.4487.00	4.11	l1.4488.00	0.25	n.s	n.s	n.s	

A: Iodine index - B: Melting point - C: Refractive index - M: mean SD: Standard Deviation - O-P: omental-perirenal, O-S: omental- subcutaneous, P-S: Perirenal-Subcutaneous. The values are:mean+standard deviation from 4 animals in each case. For omental:a-b,a-c,i-k,i-l:P<0.05, a-d,i-j:P<0.01. For perirenal:a-b,i-j:P<0.01 a-c,i-k,i-l:P<0.05, a-d:P<0.001. For subcutaneous: a-b:P<0.05, a-d,i-j,i-k,i-l:P<0.01, e-f,e-g,e-h:P<0.001. The values of SD of C must be multiply by 10-4.

ohydrate diet. There were not differences between protein and fat diets for these tissues.

In subcutaneous adipose tissue the iodine index was the highest with carbohydrate diet and the least with fat diet. There were not differences between alfalfa hay and protein diets.

The melting point, with alfalfa hay, was the highest in perirenal adipose tissue than in the others. With fat, carbohydrate and protein diets the least value of this index was observed in subcutaneous adipose tissue.

The melting point in omental and perirenal adipose tissue were not diet dependent, but in subcutaneous the highest value was observed with alfalfa hay. Between the other diets, the value of melting point not changed.

The refractive index, with alfalfa hay diet, the least value was observed in subcutaneous adipose tissue. With fat and protein diets, the values of this index were the highest in subcutaneous adipose tissue. With carbohydrate diet, there were not differences between the three adipose tissues studied.

DISCUSSION

There are not previous reports about the composition of subcutaneous adipose tissue of goats submitted to different dietary treatments.

In general, this study has revealed differences in the saturated and unsaturated fatty acids content in subcutaneous adipose tissue in relation to the dietary treatment.

With high fat supplemented diet there was a grand accumulation of saturated fatty acids and with high carbohydrate supplemented diet it was accumulated major amount of unsaturated fatty acids.

Some physicochemical properties of the deposited fat were very affected by these different treatments. The value of these properties are associated to the quantity and quality of saturated or unsaturated fatty acids, present in the deposited fat.

Several studies have demonstrated that the level of nutrition influences fat content in cattle (9). The quantity of forage in a diet alters the saturation degree of fatty acids in the fat depot. The animals fed with forage have more saturated fatty acids in their fat than those fed with high carbohydrate diet.

The time of feeding is very important in determining the composition of the fat depot. The most changes are observed

in the experiment effected with animals fed during two consecutive years with the diet in assaying (10).

Our results have showed that a high carbohydrate supplemented diet facilitates the fat deposition in subcutaneous adipose tissue of goat compared with a high fat or a high protein diet.

There are diverse causes that could justify the nature of the deposited fatty acids. They are associated to the fatty acids composition of the diets, the action of the ruminal flora on these fatty acids and the activities of desaturases present in different tissues (11).

The fatty acids composition of fat depot is very important because it contributes to the palatabilidad of the meat. The unsaturated fatty acids have a positive effect on the flavor meat.

We can conclude that to obtain goat meat with more acceptability in the market, the animal must be fed with carbohydrate supplemented diet, because in this condition the subcutaneous fat depot contains more amount of unsaturated fatty acid and consequently diminished melting point and high iodine index.

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REFERENCES

- 1- Sumida,D.M., Voght,D.W., Cobb,E.T., Iwanaga,I.I. and Reimet ,D. Effect of breed type and feeding regime on fatty acid composition of certain bovine tissues. J. Anim. Sci., 35, 1058-1063,(1972).
- 2- Westerling,D.B. and Hedrick H.B. Fatty acid composition of bovine lipids as influenced by diet, sex and anatomical location and relationship to sensory characteristics. J. of Anim. Sci., 48, 1343-1348,(1979).
- 3- García,P.T., Casal,J.J. and Parodi,J.J. Crossbreeding and quality of steer fat depots. Prod. Anim. (Bs.As. Argentina), 10, 427-434,(1983).
- 4- Melton,S.L. Amiri,N. Davis W. and Backus W.R, Flavor and chemical characteristic of ground beef from grass, forage

NUTRITION REPORTS INTERNATIONAL

-grain and grain-finished steers. J. of Anim. Sci., 55, 77-83, (1982).

- 5- Official Methods of Analysis. Association of Official Analytical Chemist, 12th ed. Whashington D.C. (1975).
- 6- Folch J., Lees M. and Sloane Stanley G.H. A simple method for the isolation and purification of total lipids from animal tissues. J.Biol.Chem., 226, 497-509, (1957).
- 7- De Schrijver R. and Privett O.S. Effects of dietary long chain fatty acids on the biosynthesis of unsaturated fatty acids in the rat. Lipids, 17, 27-34, (1982).
- 8- Winer B.J. Statistical principles in experimental design. Mc Graw-Hill, New York, 46-104, (1962).
- 9- Williams J.E., Wagner D.G., Walters L.E., Horn, G.W. Waller G.R., Sims P.L. and Guenther J.J. Effect of production systems on performance, body composition and lipid and mineral profile of soft tissue in cattle. J. of Anim. Sci. 57, 1020-1028, (1983).
- 10- Marmer W.N., Maxwell R.J. and Williams J.E. Effects of dietary regimen and tissue site on bovine fatty acid profiles. J. of Anim. Sci., 59, 109-121, (1984).
- 11- Vernon R.G. Lipid metabolism in the adipose tissue of ruminant animals. Prog. Lipid Res., 19, 23-106, (1980).

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EFFECTS OF CHROMIUM AND ASCORBATE DEFICIENCIES ON GLUCOSE TOLERANCE AND SERUM CHOLESTEROL OF GUINEA PIGS

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ABSTRACT

Chromium and ascorbate deficiencies were investigated in guinea pigs. Weanling animals were fed chromium deficient (-Cr) or chromium adequate (+Cr) casein-based diets (0.1 or 1.9 ppm Cr) and were supplemented with 10 mg ascorbic acid/day for 14 weeks. After 14 weeks half of each group continued to receive 10 mg ascorbic acid/day (+C) while the other half received no ascorbic acid (-C). Oral glucose tolerance tests (1 g glucose/kg body weight) were conducted. At week 16, 120 minutes after the glucose load, plasma glucose concentrations (mg/dl) were 116 ± 20 , 133 ± 21 , 129 ± 13 , and 188 ± 14 for groups +Cr+C, +Cr-C, -Cr+C, and -Cr-C respectively. Plasma glucose of group -Cr-C was higher than all other groups ($p < 0.05$). At week 16 mean plasma cholesterol levels were significantly higher in group -Cr-C than in group +Cr+C (89 ± 4 vs. 50 ± 7 mg/dl; $p < 0.05$). Combined chromium and ascorbic acid deficiency resulted in impaired glucose tolerance and elevated plasma cholesterol.

INTRODUCTION

Both chromium and ascorbate deficiencies have been observed individually to contribute to impaired glucose tolerance and to elevated serum cholesterol. Mertz and Schwarz (1) reported in 1955 that rats fed a Torula yeast diet developed impaired glucose tolerance and that the impairment was prevented by addition of Brewer's yeast to the diet. They later identified chromium as the active element in Brewer's yeast (2). Mertz and coworkers have suggested that chromium enhances the effect of insulin on glucose transport from the bloodstream into the tissues (3).

Evidence for chromium involvement in cholesterol homeostasis has been equivocal. Higher serum cholesterol levels have been reported in rats fed low chromium diets (4,5). Abraham and colleagues demonstrated significantly less atherosclerotic plaque in rabbits fed 1.5% cholesterol diets and injected daily with 20 μ g potassium chromate than in the group

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which did not receive chromium (6). In a subsequent study of rabbits fed 1% cholesterol diets, Abraham and coworkers reported consistently, but not significantly, lower cholesterol levels in chromium-treated rabbits compared with those of controls (7). Preston and colleagues, however, in a short study (4) found no hypercholesterolemia or hyperglycemia in guinea pigs fed a low chromium diet for five weeks (8). In some human studies chromium supplementation has produced a significant reduction in serum cholesterol, but in other studies supplementation has not contributed to decreased cholesterol levels (9).

In chronic ascorbic acid deficiency Ginter found that guinea pigs had lower bile acid production and higher liver and serum cholesterol concentrations than controls (10). Several laboratories have observed decreased catabolism of cholesterol to bile acids in ascorbic acid deficient guinea pigs (11-13). Ginter has suggested that ascorbic acid deficiency interferes with cholesterol homeostasis by inhibiting cholesterol 7- α hydroxylase, a rate limiting step in cholesterol catabolism (11).

A role for insulin in ascorbic acid transport in human red blood cells has been reported by Mann and Newton (14). They also found that transport of ascorbic acid was competitively inhibited by glucose and that hyperglycemia impaired intracellular ascorbic acid availability. Diabetic rats have shown decreased tissue ascorbate concentrations (15) and diabetic humans beings have been reported to have low tissue ascorbate levels (16).

This study was designed to investigate whether or not chromium and ascorbic acid depletion would act synergistically to create impaired glucose tolerance and elevated plasma cholesterol in guinea pigs.

METHODS

Male Hartley guinea pigs¹ weighing approximately 200 g were randomly assigned to negative control (-Cr) or positive control (+Cr) diets (Table I). Diets and mineral mix were formulated in our laboratory after testing individual components for chromium contamination. The +Cr diet was identical to the -Cr diet except that 2.0 ppm chromium as CrCl_3 was added to the positive control. Diets were pelleted to increase acceptability. Both +Cr and -Cr groups were initially supplemented with approximately 10 mg/day ascorbic acid in their drinking water.

Animals were housed in plastic cages on plastic egg crating material. Food and distilled deionized water were available ad libitum in ceramic cups.

After 14 weeks the positive (+Cr) and negative (-Cr) control groups were each subdivided into 2 groups. The +Cr-C and the -Cr-C groups were depleted of ascorbic acid for 14 days before the oral glucose tolerance test and plasma cholesterol analysis at week 16 while the +Cr+C and the -Cr+C groups continued to receive 10 mg ascorbic acid per day.

Fasting blood samples were taken from the guinea pigs' toenails (17) before they were intubated with glucose (1g/kg

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TABLE I

Composition of the basal diet¹

Component	g/kg
Casein	300
Arginine	3
Dextrose	370
Celufil	150
Corn oil	70
Vitamin mix ² (without ascorbic acid)	22
Potassium acetate	35
Mineral mix ³ (without chromium)	50

¹ By analysis the basal diet contained 0.106 ppm Cr.

² The vitamin mix contained per kg (in g except as noted)
 α -tocopherol 5.0; choline chloride 75.0; d-calcium
 pantothenate, 3.0; inositol, 5.0; menadione, 2.25; niacin,
 4.5; paraaminobenzoic acid, 5.0; pyridoxine HCl, 1.0;
 riboflavin, 1.0; thiamine HCl, 1.0; vitamin A acetate, 900,000
 units; calciferol (D₂), 100,000 units; biotin, 20 mg; folic
 acid, 90 mg; and vitamin B₁₂, 1.35 mg.

³ The mineral mix contained (g/kg) CaHPO₄, 600.0; NaCl, 80.0;
 MgO, 100.0; ZnCO₃, 0.9; MnCO₃, 1.8; CuCO₃·Cu(OH)₂·H₂O, 0.25;
 KIO₃, 0.035; NaSeO₃·5H₂O, 0.0075; and FeSO₄, 0.25.

body weight) as a 50% w/v solution. After the glucose load, blood samples were taken at 30, 60, 90, and 120 min.

Glucose was determined by a coupled enzymatic colorimetric procedure utilizing horseradish peroxidase, glucose oxidase, and O-dianisidine (18). Plasma cholesterol was determined using an enzymatic colorimetric method (19).

Chromium content of diets was analyzed with a Perkin Elmer 5000 atomic absorption spectrophotometer with graphite furnace and Zeeman background correction. Diet samples (250 mg) were ashed in 12 x 75 mm acid-washed borosilicate glass tubes. Ashing temperature was raised 50° C per hour from 100 to 300° C. Samples were then ashed overnight at 500°C. After cooling, 0.1 ml H₂O, 0.1 ml HNO₃ and 0.1 ml 50% H₂O₂ were added and the samples were evaporated to dryness on a hot plate at 105°C. Samples were then ashed overnight at 500°C. Addition of H₂O, HNO₃ and H₂O₂ and ashing were repeated until ash was white.² Care³ was taken to avoid sample contamination or loss. The -Cr diet was analyzed to contain 0.106 ppm Cr and the +Cr diet to contain 1.910 ppm Cr.

A generalized linear model of the Statistical Analysis System (20) was used to analyze the 2 x 2 factorial design and the Duncan's multiple range test was utilized at the 0.05 alpha level to determine significant differences among groups.

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RESULTS AND DISCUSSION

Growth

Body weight was not affected by the chromium depletion diet (Table II). After 2 weeks of ascorbic acid depletion (week 16),

TABLE II

Body weights at 16 weeks of guinea pigs depleted of chromium and/or ascorbic acid and controls^{1,2}

Group ³	No. of Animals	Body Weight
-Cr-C	8	697 ± 29 ^a
-Cr+C	6	723 ± 61 ^a
+Cr-C	6	738 ± 27 ^a
+Cr+C	7	655 ± 27 ^a

¹ Mean ± SEM

² Means in a column with common supercripts are not significantly different (p > 0.05) using Duncan's multiple range test.

³ -Cr-C = Chromium deficient, ascorbic acid deficient; -Cr+C = Chromium deficient, ascorbic acid adequate, +Cr-C = Chromium adequate, ascorbic acid deficient, +Cr+C = Chromium adequate, ascorbic acid adequate.

growth rates slowed and body weight dropped precipitously soon thereafter. However, weights were not yet significantly different at week 16.

Glucose Tolerance

Results of glucose tolerance tests at 7, 10, and 13 weeks were not significantly affected by chromium depletion and are not shown. Preston and Dowdy (8), likewise, found no hyperglycemia in guinea pigs fed a low chromium diet for 5 weeks. But, in our study with the longer feeding period and the removal of ascorbic acid from the diet, the impaired glucose tolerance became apparent at 16 weeks.

At week 16 the fasting glucose level of the group depleted of both ascorbic acid and chromium (-Cr-C) tended to be higher than other groups (Table III). Values at 30 and 60 minutes after the glucose load (not shown) were affected by individual absorption rates and no differences were seen between groups. Ninety minutes after the glucose load, the -Cr-C group was higher than both groups which received adequate ascorbic acid. At 120 minutes after the glucose load, group -Cr-C was higher

TABLE III

Fasting plasma glucose and glucose at 90 and 120 minutes after a glucose load (1g/kg body weight) of guinea pigs fed for 16 weeks and depleted of chromium and/or ascorbic acid and controls 1,2.

Group ³	No. of animals	Glucose at		
		Fasting	90 min	120 min
-Cr-C	7	131 ± 4 ^a	245 ± 11 ^a	188 ± 14 ^a
-Cr+C	5	97 ± 16 ^a	172 ± 17 ^b	129 ± 13 ^b
+Cr-C	5	99 ± 14 ^a	190 ± 26 ^{ab}	133 ± 21 ^b
+Cr+C	6	102 ± 10 ^a	164 ± 27 ^b	116 ± 20 ^b

¹ Mean ± SEM.

² Means in a column not sharing a common superscript are significantly different ($p < 0.05$) using Duncan's multiple range test.

³ See Table II for legend.

than all other groups.

With analysis of variance, ascorbic acid deficiency showed a stronger relationship to elevated glucose than did chromium although the glucose elevation was exacerbated in guinea pigs depleted of both chromium and ascorbate. At 120 minutes after the glucose load, the effect of chromium reached the 0.06 level of significance while the effect of vitamin C was significant at $p < 0.05$. Of course, the degree of chromium deficiency cannot be assumed to be identical to the degree of ascorbic acid deficiency. Ascorbic acid deficiency occurs rapidly and chromium deficiency tends to develop more slowly. Animals were depleted of chromium for 16 weeks and of ascorbic acid for 2 weeks but were probably in a borderline Cr deficiency state while their vitamin C deficiency became severe only a few days after the 16-week test.

Plasma Cholesterol

At 16 weeks the plasma cholesterol of the group depleted of chromium and ascorbic acid (-Cr-C) was significantly higher than both groups fed adequate chromium, and the -Cr+C group was also significantly higher than the +Cr+C group (Table IV). By analysis of variance, the effect of chromium depletion upon blood cholesterol levels was highly significant ($p < 0.0003$).

Other laboratories have reported elevated total serum cholesterol with ascorbic acid deficiency and with chromium deficiency but it is not clear why chromium and ascorbate deficiencies appear synergistic. At the gastrointestinal level ascorbate may facilitate the absorption of chromium as it does iron, or ascorbate depletion may inhibit chromium absorption or cholesterol reabsorption. If insulin is required for the uptake

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TABLE IV

Plasma cholesterol at 16 weeks of guinea pigs, depleted of chromium and/or ascorbic acid and controls ^{1,2}

Group ³	No. of Animals	Cholesterol
		mg/dl
-Cr-C	8	89 ± 4 ^a
-Cr+C	6	75 ± 8 ^{ab}
+Cr-C	6	60 ± 7 ^{bc}
+Cr+C	7	50 ± 7 ^c

¹ Mean ± SEM

² Means in a column not sharing a common superscript are significantly different ($p < 0.05$) using Duncan's multiple range test.

³ See Table II for legend.

of ascorbate into certain tissues, chromium deficiency may impair ascorbate uptake as it does that of glucose. Decreased intracellular ascorbate could decrease 7 α -hydroxylation of cholesterol with resultant hyperglycemia. Further studies are needed to clarify mechanisms of chromium-ascorbate interactions.

References

1. Mertz, W. and Schwarz, K. Impaired intravenous glucose tolerance as an early sign of dietary necrotic liver degeneration. Arch Biochem Biophys 58:504-506 (1955).
2. Schwarz, K. and Mertz, W. Chromium (III) and the glucose tolerance factor. Arch Biochem Biophys 85:292-295 (1959).
3. Mertz, W., Toepfer, E.W., Roginski, E.E. and Polansky, M.M. Present knowledge of the role of chromium. Federation Proc 33:2275-2280 (1974).
4. Schroeder, H.A. Serum cholesterol levels in rats fed thirteen trace elements. J Nutr 94:475-480 (1968).
5. Schroeder, H.A. Serum cholesterol and glucose levels in rats fed refined and less refined sugars and chromium. J Nutrition 97:237-242 (1969).
6. Abraham, A.S., Sonnenblick, M., Eini, M., Shemesh, O., and Batt, A.P. The effect of chromium on established atherosclerotic plaques in rabbits. Am J Clin Nutr 33:2294-2298 (1980).
7. Abraham, A.S., Sonnenblick, M. and Enin, M. The effect of

NUTRITION REPORTS INTERNATIONAL

- chromium on cholesterol-induced atherosclerosis in rabbits. *Atherosclerosis* 41:371-397 (1982).
8. Preston A.M., Dowdy R.P., Preston M.A., and Freeman, J.N. Effect of dietary chromium on glucose tolerance and serum cholesterol in guinea pigs. *J Nutr* 106:1391-1397 (1976).
 9. Borel, J.S. and Anderson, R.A. Chromium. In: *Biochemistry of the Essential Ultratrace Elements*, Vol. 3. Frieden, E. (Ed.), Plenum Press, New York 1984 pp. 175-199.
 10. Ginter, E., Cervin, J., Nemec, R., and Mikus, L. Lowered cholesterol catabolism in guinea pigs with chronic ascorbic acid deficiency. *Am J Clin Nutr* 24:1238-1245 (1971).
 11. Ginter, E., Bobek, P., Kubec, F., Vozar, J., and Urbanova, D. Vitamin C in the control of hypercholesterolemia in man. *Int J Vit Nutr Res* 23:137-152 (1982).
 12. Harris, W.S., Kottke, B.A., and Subbiah, M.T.R. Bile acid metabolism in ascorbic acid-deficient guinea pigs. *Am J Clin Nutr* 32:1837-1841 (1979).
 13. Holloway, D.E., and Rivers, J.M. Influence of chronic ascorbic acid deficiency and excessive ascorbic acid intake on bile acid metabolism and bile composition in the guinea pig. *J Nutr* 111:412-424 (1981).
 14. Mann, G.V., and Newton, P. The membrane transport of ascorbic acid. *Ann NY Acad Sci* 258:243-252 (1975).
 15. Zebrowski, E.J., Bhatnager, P.K. and Brunka, J.R. Ascorbic acid status of streptozotocin diabetic and insulin treated rats. *J Dental Res* 55:B145 (1976).
 16. Ginter, E., Zdichynec, B., Holzerova, O., et al. Hypocholesterolemic effect of ascorbic acid in maturity-onset diabetes mellitus. *Int J Vit Nutr Res* 48:368-733 (1978).
 17. Lehnert, J.P. New method for bleeding guinea pigs. *Tech Bull Med Technol* 37:52-53 (1967).
 18. Sigma Chemical Company. The enzymatic colorimetric determination of glucose in whole blood, plasma or serum at 425-475 nm. St. Louis, MO: Sigma Chemical Co., No. 510 (1978).
 19. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. Enzymatic determination of total serum cholesterol. *Clin Chem* 20:470-475 (1974).
 20. SAS Institute. SAS User's Guide 1979 Edition. Raleigh NC: SAS Institute, Inc. (1979).
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EFFECTS OF VITAMIN E DEFICIENCY ON GROWTH AND ALKALINE PHOSPHATASE ACTIVITIES IN SERUM AND BONES OF DEVELOPING RATS

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ABSTRACT

Twenty one-day old male weanling rats were maintained for five weeks on a diet deficient in vitamin E. Compared to rats fed a diet containing adequate vitamin E, the weights of their bones were considerably reduced and the incisor growth rate was reduced by 25%. There were qualitative and quantitative differences in the composition of lipids extracted from the femur of the rats. In vitamin E deficiency, the acidic phospholipids were considerably reduced, whilst cholesterol and phosphatidyl choline were considerably increased in proportion. Vitamin E deficiency was also characterized by a reduction in the activities of alkaline phosphatase in the bones and liver and an increase in its activity in the serum. It is suggested that vitamin E may play a role in the mineralization of bones and that its deficiency may cause the initiation of bone and liver diseases.

INTRODUCTION

Rats fed vitamin E deficient diets cease to grow after a period of time and body weight declined until death. Vitamin E deficiency also inhibits growth whilst its excess causes fatigue (1,2,3).

It has been reported that the concentration of lipids in skeletal muscle and serum increased in vitamin E deficient animals (4). The lipid peroxides accumulate rapidly in vitamin E deficiency (5) and this effect can be reduced by administration of vitamin E (6). Lesions in skeletal muscles are universal in all laboratory animals subjected to vitamin E deficiency. Later changes such as myofibrillar disruption, accumulation of lipid droplets in the sarcoplasm and intramitochondrial calcification are considered to be secondary in nature (7).

There is evidence to suggest that bone alkaline phosphatase plays a role in the calcification of bones. The activity of the enzyme in bone appears to correlate roughly with the number of identifiable osteoblasts (8,9). Righetti and Kaplan (10) reported that bone is a major source of alkaline phosphatase in the serum of the normal rat.

There appears, however, to be no information on the effect of vitamin E deficiency on bone and tooth growth or on bone lipid composition. This experiment was therefore designed to investigate the effect of vitamin E deficiency on the development and lipid composition of bones. The changes in serum, liver and bone alkaline phosphatase activities were also investigated.

MATERIALS AND METHOD

Animals and diets:

Twenty male Wistar rats were divided into two groups of ten which were maintained on the following diets:

- (a) control diet, (this is the same as the deficient diet but contains 0.4% ergocalciferol)
- (b) diet deficient in vitamin E.

The composition of the deficient diet is shown in Table 1. The diets and water were given ad libitum. The animals were placed on the diet at 21 days of age (average weight 26.5g) blocked by weight and randomly assigned to the different dietary treatments. Incisor growth rate was measured by marking the tooth at the gingival margin with a file and the rate of movement of the mark measured (11). All rats were fed their respective diets daily and weighed weekly. The diets consumed were measured daily. At the end of the fifth week, the rats were killed by decapitation. Blood was collected in specimen tubes and after clotting it was centrifuged at 2000 r.p.m. for 10 minutes and the serum was separated and stored in the refrigerator at 0°C. The teeth and bones were removed, cleaned of surrounding soft tissue and dried. The weights and lengths of the bones were recorded.

Lipid analysis:

Bones were freeze-dried, pulverised and the lipids extracted and purified as previously described (12,13). The lipids were separated into individual neutral lipids and quantified as previously described (14-17).

Analysis of calcium and phosphorous:

Known weights of pure bone samples obtained as described above were dissolved in 5N HCl (18) and aliquots analysed for calcium (19) and phosphorous (20). Ammonium purpurate (murexide) solution (60mg/100ml in 70% ethanol) was used to determine the degree of mineralization of the bone samples (21).

TABLE 1 Composition of diets

Ingredients	% weight
Casein ¹	25.0
DL-methionine	0.4
Corn starch	51.6
Cellulose	4.0
Sucrose	10.0
Corn oil	4.0
Mineral mix ²	4.0
Vitamin mix ³	1.0

- 1 Commercial casein (vitamin free) was extracted with ethanol and chloroform/methanol to remove any fat soluble contaminant. It contained approximately 91.5% crude protein (7).
- 2 Mineral mix (g/kg diet): CaCO_3 , 6.54; CuSO_4 , 0.0072; KI, 0.0016; NaCl, 4.32; ZnCO_3 , 0.0176; Ca(OH)_2 , 19.69; Na_2SeO_3 , 0.017; KH_2PO_4 , 3.11; MgSO_4 , 1.13; KHSO_4 , 7.22; MnO_2 , 0.0955; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.001, $\text{Fe}_2(\text{SO}_4)_3$, 0.38.
- 3 Vitamin mix (mg/kg diet): L-Ascorbic acid, 1000; calcium Panthothenate, 66.6; Niacin 99.89; Pyridoxin-Hcl, 22.2; Riboflavin, 22.2; Thiamin-Hcl, 22.2; Folic acid, 2; I-inositol, 110.9; PABA, 110.9; Vitamin B_{12} , 0.03; Biotin, 0.44; choline chloride 1665; vitamin A, 11; Vitamin D, 2220 i.u., menadione, 0.10.

Enzyme assay

The alkaline phosphatase from femur samples was prepared by the method of Lai et al. (22) and the activities in bone, serum and liver samples were assayed by King-Armstrong's method (23). To obtain the activity of alkaline phosphatase in m.I.U., one King-Armstrong unit was multiplied by 7.08.

Statistical analysis

Analysis of variance were carried out to determine the statistical significance of results.

RESULTS AND DISCUSSION

Animals fed the vitamin E deficient diet showed a significant ($P/0.05$) decrease in body weight gain compared with the controls (Fig.1). Yang and Desai (24) observed similar growth retardation. The vitamin E deficient rat group consumed more food than the control rats and so the reduction in body weight could be attributed mainly to the effect of the vitamin E deficiency.

Vitamin E deficiency had no significant effect on the length of femur, tibia, scapula and humerus during the period of the experiment (Table 2). Chan and Hegarty (2) observed, using bone length as an index of muscular length, no significant difference in muscle length of vitamin E deficient rabbits.

Compared to the controls, the weights of femur, tibia and scapula were significantly ($P/0.05$) lower in rats fed the vitamin E deficient diet. The growth rate of the incisors was also reduced in animals fed the deficient diet (Table 2). The results of calcium and phosphorous analysis (Table 3) showed that the animals fed the control diet and the diet deficient in vitamin E had molar ratios of Ca/P of 1.62 to 1.63 and 1.46 to 1.52 respectively. A molar ratio of calcium to phosphorous of 1.5 - 1.63 is considered to occur in bone mineral (apatite) depending on how it is formed (25). Although the present results show that in the deficiency state, bone mineral was still apatite in nature, the molar calcium-to-phosphorous ratio in the deficient animals was significantly reduced ($P/0.01$) when compared with those fed the control diet. This would indicate that less calcium in relation to phosphorous had been deposited when animals were fed the deficient diet.

The murexide staining, however, showed that the bones of animals maintained on a diet deficient in vitamin E were hypomineralized. This indicates impaired or immature mineralization. It might have been induced either by a retardation in the development of the rats or caused by

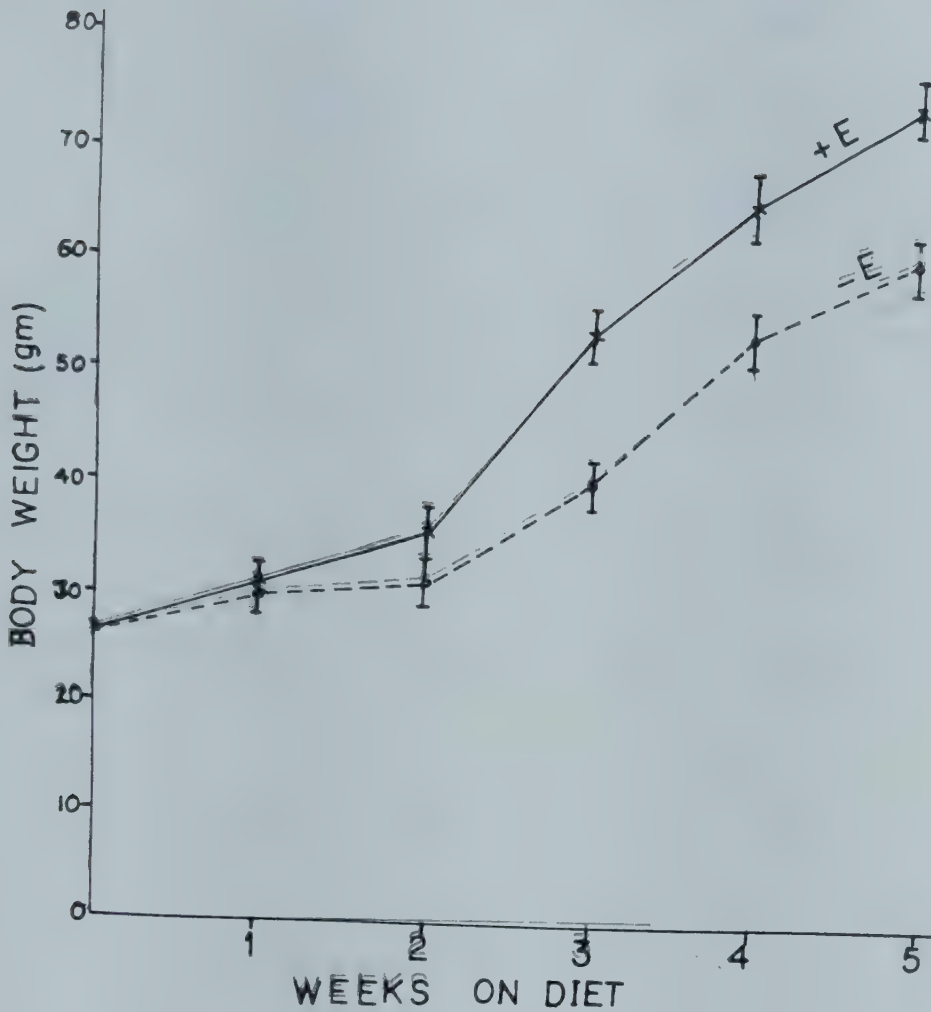


Fig. 1. Growth of rats fed a diet with (+E) and without (-E) vitamin E supplementation.

malformed organic matrix.

The total bone lipid composition was significantly ($P/0.05$) elevated in the vitamin E deficient group (Table 4). Mason (7) observed that vitamin E deficiency may lead to accumulation of lipid droplets in the sarcoplasm. Other investigators have also shown that the concentration of lipids in skeletal muscle and serum increased in animals on vitamin E deficient diets (4,26). A significant increase ($P/0.05$) in cholesterol and phosphatidylcholine was observed.

TABLE 2 Effect of vitamin E deficiency on the length and weight of bones and incisor growth rate of developing rats. The result are the mean values from ten analyses \pm S.E.M.

Treatment	Femur	Tibia	Scapula	Humerus	Incisor growth rate (mm/day)
Length of bones (cm)					
+Vitamin E	2.06 \pm 0.04 \pm	2.31 \pm 0.05 \pm	1.42 \pm 0.03 \pm	1.56 \pm 0.03 \pm	0.36 \pm 0.02 \pm
-Vitamin E	2.04 \pm 0.02 \pm	2.30 \pm 0.03 \pm	1.47 \pm 0.03 \pm	1.59 \pm 0.03 \pm	*0.27 \pm 0.01 \pm
Weight of bones (mg)					
+Vitamin E	91.00 \pm 2.40 \pm	75.70 \pm 2.60 \pm	32.25 \pm 1.01 \pm	42.90 \pm 3.31 \pm	-
-Vitamin E	*84.60 \pm 1.70 \pm	*70.40 \pm 1.30 \pm	*27.30 \pm 0.64 \pm	40.03 \pm 2.90 \pm	-

*Significantly different from control at $P/0.05$.

We found that the proportions of acidic phospholipids phosphatidylserine, phosphatidyl inositol, phosphatidic acid and diphosphoglycerol were significantly ($P/0.05$) reduced in the vitamin E deficient group (Table 4). The acidic phospholipids have been implicated in the calcification process (11, 26, 27).

It has also been shown that when rats were fed a diet deficient in essential fatty acids the acidic phospholipids were considerably reduced in proportion and a higher percentage of lipid was extracted from their bones (26). It is possible that vitamin E may be necessary for the metabolism of the essential fatty acids that have been implicated in the growth and mineralization of bone. Essential fatty acids are normally converted to prostaglandins. It is possible that vitamin E may have a role to play in the

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TABLE 3 Calcium and phosphorous analysis of calcified tissues from rats fed different diets. (Mean values of ten animals expressed as percentage dry weight of respective tissues \pm S.E.M.)

Tissue	+Vitamin E	-Vitamin E
Femur		
Calcium %	36.21 \pm 0.51	31.70 \pm 0.26
Phosphorous %	18.20 \pm 0.02	16.10 \pm 0.22
Ca/P molar ratio	1.62 \pm 0.03	* 1.52 \pm 0.02
Tibia		
Calcium %	31.20 \pm 0.22	27.20 \pm 0.20
Phosphorous %	14.80 \pm 0.10	14.00 \pm 0.06
Ca/P molar ratio	1.63 \pm 0.02	* 1.50 \pm 0.03
Scapula		
Calcium %	36.00 \pm 0.37	30.09 \pm 0.23
Phosphorous %	17.20 \pm 0.10	15.90 \pm 0.07
Ca/P molar ratio	1.62 \pm 0.01	* 1.46 \pm 0.04

*Significant difference from control at $P/0.01$.

synthesis of prostaglandins which are necessary for normal bone mineralization (27, 28).

Femur and liver alkaline phosphatase was considerably reduced and serum alkaline phosphatase was significantly ($P/0.05$) elevated in the animals fed the deficient diet (Table 5). Elevations of serum alkaline phosphatase have been reported in patients with bone disorders (29, 30).

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TABLE 4 Rat femur lipids. Values are expressed as percentage dry weight and are the mean values from ten femurs for each dietary group (analysed in duplicate) \pm S.E.M.

Analysis	+Vitamin E	-Vitamin E
Monoacylglycerol	1.7 \pm 0.4	1.2 \pm 0.3
Diacylglycerol	1.8 \pm 0.1	1.3 \pm 0.3
Cholesterol	19.6 \pm 2.0	25.8 \pm 1.9
Free fatty acid	4.8 \pm 0.5	5.2 \pm 0.3
Triacylglycerol	12.0 \pm 1.0	11.7 \pm 0.8
Cholesterol ester	13.4 \pm 0.8	12.5 \pm 0.5
Phosphatidylserine	4.9 \pm 0.1	4.2 \pm 0.1
Phosphatidyl inositol	5.9 \pm 0.2	3.7 \pm 0.5
Sphingomyelin	3.8 \pm 0.1	4.6 \pm 0.4
Phosphatidylcholine	14.6 \pm 0.1	18.4 \pm 0.5
Phosphatidyl ethanolamine	7.3 \pm 0.6	5.8 \pm 0.6
Phosphatidic acid	5.0 \pm 0.1	2.7 \pm 0.6
Diphosphoglycerol	5.2 \pm 0.8	2.9 \pm 0.6
Total Neutral lipid (mg/g)	1.92 \pm 0.1	2.20 \pm 0.1
Total Phospholipid (mg/g)	1.69 \pm 0.4	1.61 \pm 0.6
Total lipid (mg/g)	3.61 \pm 0.4	3.81 \pm 0.8
Total lipid (% dry weight of bone)	0.36 \pm 0.006	0.38 \pm 0.007

Such enzyme elevation often correlates with severe bone disorders. This has been associated with increased osteoblastic activity and with disorders that impede bile flow. In most disorders affecting the liver, an increase in serum alkaline phosphatase resulted (31, 32).

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TABLE 5 Effect of diets on femur, liver and serum alkaline phosphatase activities. (The values are means of 10 animals in each case \pm S.E.M.)

Treatment	m.I.U/g tissue		m.I.U/L
	Femur	Liver	Serum
+Vitamin E	193.00 \pm 0.27	101.24 \pm 0.10	117.32 \pm 0.50
-Vitamin E	*108.25 \pm 0.29	* 64.14 \pm 0.9	*205.04 \pm 0.74

*Significant difference from control at $P \leq 0.05$.

The electrophoretic pattern of both the liver and bone alkaline phosphatase did not give any clue as to the origin of the serum alkaline phosphatase. The present results, however, showed that bone alkaline phosphatase was more drastically reduced than the liver alkaline phosphatase in vitamin E deficiency.

It is therefore considered that the alkaline phosphatase activity of serum might be due to enzyme of bone origin.

The decrease in bone and liver alkaline phosphatase and the increase in serum alkaline phosphatase in the rats on the vitamin E deficient diet might be an indication of the initiation of bone and liver disease.

The present study has confirmed that vitamin E is essential for the normal growth and mineralization of the bone in the rat. It is possible that the deficiency may lead to abnormal bone calcification.

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NUTRITION REPORTS INTERNATIONAL

REFERENCES

1. Briggs, M. - Vitamin E supplements and fatigue.
N. Eng. J. Med. 290, 579-580 (1974).
2. Chan, A.C. and Hegarty, P.V.J. - Morphological changes in skeletal muscles in vitamin E deficient and re-fed rabbits. Br. J. Nutr. 38, 361-370 (1977).
3. Cohen, M.M. - Effects of vitamin E: Good and Bad.
N.Eng. J. Med. 289, 980 (1973).
4. Alfin-Slater, R.B. - Relation of vitamin E to lipid metabolism. Am.J.Clin.Nutr. 8 445 (1960).
5. Century, B. and Horwitt, M.K. - Role of diet lipids in the appearance of dystrophy and creatinuria in the vitamin E deficient rat. J. Nutr. 72 357-367 (1960).
6. Prodouz, K.N. and Navari, R.M. - Effects of vitamins A and /E on rat tissue lipids. Nutr. Res. Int 11 17-28 (1975).
7. Mason, K.E. - Effect of nutritional deficiency on muscle. In The Structure and Function of Muscle. Edited by Bourne, G.H., 2nd Ed. Vol. 4 pp. 155-206. Academic Press, New York (1973).
8. Jeffre, E.M. - Phosphatase activity in the limb bones of monkeys (*Lagothrix lumboldti*) with hyper-parathyroidism. J. Clin. Pathol. 15, 99-111 (1962).
9. Teaford, M.E. and White, A.A. - Alkaline phosphatase and osteogenesis in vitro. Proc. Soc. Expt. Biol. Med. 117, 541-546 (1964).
10. Righetti, A.B.B. and Kaplan, M.M. - The origin of the serum alkaline phosphatase in normal rats. Biochim. Biophys. Acta 230, 504-509 (1971).
11. Prout, R.E.S. and Atkin, E.R. - Effect of diet deficient in essential fatty acid on fatty acid composition of enamel and dentine of the rat. Arch. Oral Biol. 18, 538-590 (1973).
12. Prout, R.E.S.; Odutuga, A.A. and Tring, F.C. - Lipid analysis of rat enamel and dentine. Archs Oral Biol. 18, 373-380 (1973).
13. Folch, J.; Lees, M. and Sloane-Stanley, G.H. - A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226 497-509 (1957).

NUTRITION REPORTS INTERNATIONAL

14. Aments, J.S. - A rapid chemical method quantification of lipids separated by thin-layer chromatography. J. Lipid Res. 5 270-272 (1964).
15. Odutuga, A.A. - Recovery of brain from deficiency of essential fatty acids in rats. Biochim.Biophys.Acta 487 1-9 (1977).
16. Odutuga, A.A. - Long time deficiency of essential fatty acids in rats and its effect on brain recovery. Clin. exp. Pharmac. Physiol. 6 361-366 (1979).
17. Odutuga, A.A. and Prout, R.E.S. - Effect of essential fatty acid deficiency on the fatty acid composition of individual lipids from enamel and dentine of the rat. Arch. Oral. Biol. 19
18. Odutuga, A.A.; Prout, R.E.S. and Hoare, R.J. - Hydroxyapatite precipitation in vitro by lipids extracted from mammalian hard and soft tissues. Arch. Oral Biol. 19 725-728 (1975).
19. Kingsley, G.R. and Robnet, O. - New dye method for direct photometric determination of calcium. Am. J. Clin. Path. 27, 223-230 (1957).
20. Barlett, G.R. - Phosphorus assay in column chromatography. J. Biol. Chem. 234 466-468 (1958).
21. Kaufman, H.E. and Adams, E. - Water soluble chelates in histochemical staining. Science 120 723-732 (1954).
22. Lai, C.C.; Singer, L. and Armstrong, W.D. - Bone composition and phosphatase activity in magnesium deficiency in rats. J. Bone and Joint Surg. 57A, 516-521 (1975).
23. King, E.J. and Armstrong, A.R.: - Determination of serum alkaline phosphatase. Cand. Med. Assoc. J. 31, 376-381 (1934).
24. Yang, I.N.Y. and Desai, D.I. - Effect of high levels of dietary vitamin E on liver and plasma lipids and fat soluble vitamins in rats. J. Nutr. 107 1418-1426 (1977).
25. Irving, J.T. - Calcium and Phosphorous Metabolism. pp. 71-92. Academic Press, New York (1973).
26. Prout, R.E.S. and Odutuga, A.A. - Lipid composition of dentine and enamel of rats maintained on a diet deficient in essential fatty acids. Archs. Oral Biol. 19, 725-728 (1974).

NUTRITION REPORTS INTERNATIONAL

27. Odutuga, A.A. - Effect of low-zinc status and essential fatty acid deficiency on bone development and mineralization. Comp. Biochem. Physiol. 71A 383-388 (1982a).
28. Odutuga, A.A. - Effect of low-zinc status and essential fatty acid deficiency on growth and lipid composition of rat brain. Clin. & Expt. Pharmac. Physiol. 9 213-221 (1982b).
29. Marshall, M.K. - Alkaline phosphatase. Progr. in Hepathol 62 Nr. 3, 452-463 (1972).
30. Roberts, W.M. - Variations in the phosphatase activity in the blood in disease. Br. J. Exp. Path. 11 90-95 (1930).
31. Gutman, A.B. - Serum alkaline phosphatase activity in diseases of the skeletal and hepatobiliary system. A consideration of the current status. Am. J. Med. 27 875-901 (1959).
32. Kay, H.D. - Plasma phosphatase II. The enzyme in disease, particularly in bone disease. J. Biol. Chem. 89, 249-266 (1930).

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THE EFFECT OF HIGH DIETARY THIAMIN ON COPPER METABOLISM IN RATS^{1,2}

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ABSTRACT

The effect of high dietary thiamin on copper metabolism was investigated in rats fed two levels of thiamin and three levels of copper. Urine thiamin was decreased by high thiamin in low and normal copper groups but not changed in the high copper group. Liver copper was decreased by high thiamin in the low and high copper groups but increased in the normal copper group. The reverse effect was observed on plasma copper. High thiamin increased plasma ceruloplasmin and erythrocyte superoxide dismutase in the low copper group but had no effect on normal and high copper ceruloplasmin. Erythrocyte superoxide dismutase was decreased in the normal and high copper groups.

INTRODUCTION

Recently Cramer and co-workers have shown that stable complexes of thiamin with cadmium (1) and copper (2) can be prepared from water solutions. In the latter reaction Cu (II) is reduced to Cu (I). If thiamin and copper form a complex in vivo it may prove to be a new interrelationship between a vitamin and a mineral and may have several possible implications in copper metabolism.

The present study was designed to determine the effect of high dietary levels of thiamin on copper metabolism by measuring copper excretion, liver and plasma copper and activities of the copper containing enzymes ceruloplasmin (EC 1.16.3.1) and superoxide dismutase (EC 1.15.1.1) in rats fed low, normal and high levels of dietary copper.

MATERIALS AND METHODS

Animal diets. Seventy-two male weanling Sprague-Dawley rats were divided into six groups, each group with an average starting weight of 43 g. Each group was fed a modified AIN-76 purified diet (3) prepared with a copper free mineral mix (ICN Nutritional Biochemicals, Cleveland, Ohio). Copper and thiamin were added as follows: low, normal and high copper groups contained 1.5, 6 and 450 mg copper/kg diet respectively; and each group contained either the basal 6 mg thiamin HCl/kg diet or

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additional thiamin to contain 600 mg thiamin HCl/kg diet.

Experimental design. Rats were placed in individual stainless steel cages in a controlled environment. Food and deionized water containing less than 0.005 ppm copper, were given ad libitum. At the end of 4 weeks the rats were placed in metabolic cages with access to water but without food in order to prevent contamination of urine and fecal samples with food. The rats were kept in these cages for a total of 57 hours over 4 days. They were removed to their original cages for 6 hours a day to be fed between the hours of 10:00 a.m. and 4:00 p.m. Urine, collected in 1% HCl, and feces were collected and frozen until analyzed.

On day 32 the animals were anesthetized with ether and blood was taken from the dorsal aorta into a heparinized syringe. The blood was placed in heparinized capped tubes and placed on ice. The hematocrit was measured using an Autocrit centrifuge (Clay-Adams, Parsippany, N.J.) and a small amount of whole blood was frozen and stored for later hemoglobin determination. The remaining blood was centrifuged and the plasma separated. Both the red blood cells and the plasma were frozen for analysis. The livers were excised and immediately frozen for analysis.

Sample handling and analysis. The enzymatic activity of superoxide dismutases of the erythrocytes was determined according to Scudder et al. (4) and plasma ceruloplasmin activity by the method of Schosinsky et al. (5).

Hemoglobin concentrations of whole blood were determined by the Cyanomethemoglobin method (Data Medical Assoc. Inc., Arlington, TN 76011).

Copper was analyzed in urine and plasma directly using a Perkin-Elmer 303 atomic absorption spectrophotometer (6). Liver and fecal samples were wet digested with nitric acid. Samples were diluted with deionized water and copper concentrations were measured as for urine and plasma.

Statistical analysis (7) was performed to determine the mean and paired student t-test and correlation coefficients determined between all variables for normal versus high thiamin diet at all 3 copper levels.

RESULTS AND DISCUSSION

All rats gained weight up to 3 weeks of age. At 4 weeks the mean weights of the rats fed high copper were significantly lower ($p < .05$) than that of the rats fed low or normal copper (Table 1) indicating a slight copper toxicity. There was no significant difference in food intakes of any of the groups. Average food intake was 19g/day for all rats. Despite the lower weights, the rats fed high copper appeared active and healthy in all other aspects. High thiamin had no effect on the weight of the rats.

Hematocrit and hemoglobin concentrations were measured to determine if any of the rats developed anemia due to copper deficiency. There was

Table I
Body weights of rats at four weeks of age

	Normal thiamin (grams)	High thiamin (grams)
Low copper	192 \pm 5 ^{a,b}	194 \pm 5
Normal copper	191 \pm 6	188 \pm 4
High copper	169 \pm 8 ^c	174 \pm 5

Note: a) \pm SEM. b) There were 12 rats in each group. c) Statistically different ($P < 0.05$) from high thiamin group.

no difference in either mean (40.9) hematocrit or mean (12.8g%) hemoglobin in any of the groups.

Urine and fecal copper values are shown in Table II. High thiamin resulted in significantly lower urinary copper in the low and normal copper groups compared to the normal thiamin groups. Urine copper excretion was higher in the high copper group but high thiamin had no

Table II

Copper content of urine and feces

	Normal thiamin	High thiamin	Significance
<u>μg Copper/57 hour urine collection sample</u>			
Low copper	3.3 \pm 0.4 ^a (11) ^b	1.5 \pm 0.2 (7)	$P < 0.005$
Normal copper	3.0 \pm 0.3 (10)	1.8 \pm 0.2 (7)	$P < 0.05$
High copper	4.1 \pm 0.6 (8)	5.0 \pm 0.6 (7)	N.S. ^c
<u>μg Copper/g dried feces</u>			
Low copper	23.3 \pm 2.4 (9)	22.3 \pm 1.4 (11)	N.S.
Normal copper	74.3 \pm 5.4 (11)	73.3 \pm 1.4 (11)	N.S.
High copper	4130 \pm 240 (12)	3860 \pm 220 (10)	N.S.

Note: a) \pm SEM. b) The numbers in parentheses represent the number of animals in each group. c) Not significant.

statistical effect. There was no effect of high thiamin on fecal copper excretion.

The major effect of high dietary thiamin was an apparent redistribution of copper between plasma and liver (Table III). Liver copper was decreased by high thiamin in the low and high copper groups but increased in the normal copper group. The opposite was true of plasma copper which decreased in the normal copper group but increased in the low and high copper groups. Haywood and Comerford (8) reported that high dietary copper resulted in a biphasic response with an initial increase in plasma copper, then a decrease below the starting level. Klevay et al. (9) reported erratic changes in plasma copper in man on a low copper diet. Our results are consistent with these reports.

Table III

Copper content of liver and plasma

	Normal thiamin	High thiamin	Significance
<u>µg Copper/g dried liver</u>			
Low copper	10.4 + 1.0 ^a (10) ^b	7.36 + 0.99 (10)	P < 0.05
Normal copper	6.78 + 0.58 (11)	21.0 + 2.5 (10)	P < 0.001
High copper	416.0 + 50.8 (11)	169.0 + 21.7 (11)	P < 0.0005
<u>µg Copper/100 ml plasma</u>			
Low copper	38.5 + 3.4 (11)	52.00 + 4.0 (10)	P < 0.05
Normal copper	109.4 + 4.80 (12)	97.2 + 2.4 (11)	P < 0.05
High copper	67.8 + 2.0 (8)	78.7 + 2.2 (9)	P < 0.005

Note: a) + SEM. b) The numbers in parentheses represent the number of animals in each group.

Plasma ceruloplasmin activity (Table IV) was very low in the low copper-normal thiamin group but was increased 45 fold by high thiamin, possibly due to copper being made available from the liver into the plasma. High thiamin had no effect on the normal or high copper groups. Superoxide dismutase activity in erythrocytes was increased in the low copper group but decreased in the normal and high copper groups. Interpretation of superoxide dismutase activity is complicated since there are two enzymes, a copper-zinc containing enzyme and a manganese

Table IV

Enzyme activities of ceruloplasmin
and super oxide dismutase

	Normal thiamin	High thiamin	Significance
<u>Activity of ceruloplasmin U/L plasma</u>			
Low copper	0.656 + 0.260 ^a (11) ^b	29.79 + 4.3 (11)	P < 0.0001
Normal copper	72.3 + 2.8 (12)	69.6 + 3.2 (11)	N.S. ^c
High copper	33.3 + 3.9 (10)	37.8 + 2.5 (12)	N.S.
<u>Activity of superoxide dismutase U/ml erythrocytes</u>			
Low copper	379 + 25 (10)	494 + 23 (9)	P < 0.005
Normal copper	605 + 11 (11)	466 + 21 (10)	P < 0.0001
High copper	654 + 22 (10)	424 + 19 (10)	P < 0.0001

Note: a) + SEM. b) The numbers in parentheses represent the number of animals in each group. c) Not significant.

enzyme (10). The assay used in this study measured the combined activities and it is not known whether the high thiamin affected one or both of these enzymes.

The results of this experiment show a biological interrelationship between dietary thiamin and copper not previously reported. While the relevance to human disease can only be conjectured, it is interesting to speculate that the supplementation of thiamin might be beneficial to patients with Wilson's disease to remove copper from the liver; also persons with other diseases associated with copper in which copper plays an important combatant role such as rheumatoid arthritis may be inadvertently worsening the condition by taking high potency vitamins that contain thiamin levels similar to those found in this study. Further studies are necessary to elucidate the mechanism by which thiamin affects copper metabolism.

REFERENCES

1. Cramer, R., Maynard, R. and Iberg, J. A metal ion complex of vitamin B₁: The preparation and structure of Cd (Thiamin) Cl₃O.6H₂O. J. Am. Chem. Soc. 103, 76-81 (1981).

2. Cramer, R., Maynard, R. and Evangelista, R. The crystal and molecular structure of a Cu(I) complex of vitamin B₁, Cu (Thiamin) Cl₂. J. Am. Chem. Soc. 106, 111-116 (1984).
3. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 107, 1340-1348 (1977).
4. Scudder, P., Stocks, J. and Dormandy, T. The relationship between erythrocyte superoxide dismutase activity and erythrocyte copper levels in normal subjects and in patients with rheumatoid arthritis. Clin. Chim. Acta. 69, 397-403 (1976).
5. Schosinsky, K., Lehmann, H. and Beeler, M. Measurement of ceruloplasmin from its oxidase activity in serum by use of O-diaaisidine dihydrochloride. Clin. Chem. 20, 1556-1563 (1974).
6. Perkin-Elmer. Standard Conditions. In: Analytical Methods for Atomic Absorption Spectrophotometry. The Perkin Elmer Corp., Norwalk, Conn. (1968).
7. SPSS User's Guide. SPSS Inc. McGraw-Hill, New York, N.Y. (1983).
8. Haywood, S. and Comerford, B. The effect of excess dietary copper on plasma enzyme activity and on the copper content of the blood of the male rat. J. Comp. Path. 90, 233-238 (1980).
9. Klevay, L. M., Inman, L. and Johnson, L. K. Effects of a diet low in copper on a healthy man. Clin. Res. 28, 7584 (1980).
10. McCord, J., Boyle, E., Day, L., Rezzolo, L. and Salin, M. A manganese-containing superoxide dismutase from human liver. In Superoxide & Superoxide Dismutase (L. Michelson, J. McCord and I. Fredovich, eds) Academic Press, New York 1977, p. 129.

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CHANGES IN CONCENTRATIONS OF RUMEN AND BLOOD CONSTITUENTS IN EWES DURING ADAPTATION TO DIETARY UREA WITH AND WITHOUT SUPPLEMENTAL CLINOPTILOLITE

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ABSTRACT

Seven rumen-fistulated mature crossbred (Rambouillet x Finnsheep) ewes were individually fed for 5 days a complete high concentrate corn-soybean meal-alfalfa diet *ad libitum* (2 ewes) or the same basal diet (B) diluted with 1.0% urea (BU)(3 ewes) or 1.0% urea plus 2.0% clinoptilolite (BUC)(2 ewes). A jugular blood sample and a rumen fluid sample were obtained from each ewe at 9 a.m. and 3 p.m. 2 days before assignment to diets and daily for 5 days after introduction to the diets. Feed consumed by ewes fed BU was less than that of ewes fed B or BUC. Hematocrit and hemoglobin were higher in ewes fed BU or BUC than in those fed B, indicating shifts in fluid movement in and out of the cardiovascular compartment. Rumen pH was increased in BU and BUC ewes compared with B ewes. Plasma Ca was reduced by BU, but not by BUC, compared with B diet. Plasma inorganic P was reduced by BU and the reduction appeared more severe with BUC. Plasma K concentration was reduced by BU and was further reduced at the a.m. sampling periods by the BUC diet, compared with B diet. It is concluded that adaptation of ruminants to urea feeding is associated with adjustments in movement of fluids and electrolytes among body compartments and between blood and rumen lumen. Clinoptilolite, whose ammonia- and cation-binding properties appear operative at physiological pH, modulates the response of the rumen and host animal to urea during early dietary adaptation. The full significance of and the possible avenues for exploiting these properties of clinoptilolite need to be explored.

INTRODUCTION

Voluminous literature on use of urea as a nonprotein-N compound in animal nutrition was reviewed [1]. Use of clinoptilolite (a naturally occurring zeolite) as an additive to ruminant diets was reviewed [2]. Ammonium ion-binding properties of clinoptilolite (NH₄-exchange capacity of 1.88 meq/g; [3]) makes this natural zeolite attractive for use as a dietary supplement to prevent ammonia toxicity in ruminants during adaptation to diets containing nonprotein-N sources. Ammonia toxicity is associated with interference with urea cycle enzymes [4,5], elevated blood glucose and ketones [6,7], and elevated portal and systemic ammonia [8]. Portal blood ammonia concentration of rats following oral dosing with ammonium carbonate is decreased by concomitant administration of clinoptilolite [9]. Clinoptilolite reduces systemic blood ammonia concentrations in sheep [10] and cattle [11] fed urea.

Despite massive data on the effects of urea on nitrogen metabolism in ruminants [1,12,13,14,15,16], only limited information is available

on changes in rumen and plasma mineral concentrations in animals during adaptation to diets supplemented with urea. Hemken *et al.* [17] and Sweeney *et al.* [18] reported that changes in rumen acetate:propionate ratios and organic matter digestibility were associated with dietary clinoptilolite additions to diets containing urea. In addition, reductions in plasma K in the presence of clinoptilolite were noted by Sweeney *et al.* [18] in cattle and by Pond *et al.* [19] in lambs.

Purposes of this experiment were to (1) monitor short term patterns of change in rumen and blood plasma concentrations of urea-N, ammonia-N, glucose, lactate and mineral elements in mature ewes during adaptation to urea supplementation, and (2) determine effects of dietary clinoptilolite on responses to urea supplementation.

MATERIALS AND METHODS

Seven rumen-fistulated mature crossbred (Rambouillet x Finnsheep) ewes were fed a complete high concentrate corn-soybean meal-finely ground alfalfa diet *ad libitum* for several weeks prior to random assignment to three dietary treatments (Table I), consisting of the basal diet

TABLE I. COMPOSITIONS OF DIETS¹

Ingredient	Basal (B)	Basal + Urea (BU)	Basal + Urea + clinoptilolite (BUC)
			%
Corn	45.00	44.55	43.63
Soybean meal	5.00	4.95	4.86
Alfalfa hay (ground)	45.00	44.55	43.63
Salt (trace mineralized)	0.50	0.49	0.49
Bone meal	1.00	0.99	0.98
Ammonium chloride	0.50	0.49	0.49
Vitamin ADE premix	+	+	+
Durabond	2.50	2.49	2.43
Limestone	0.50	0.49	0.49
Clinoptilolite	--	--	2.00
Urea	--	1.00	1.00
Total, %	100.00	100.00	100.00

¹ Fed in meal form.

(B) (2 ewes), B plus 1% urea (BU) (3 ewes), or B plus 1% urea plus 2% clinoptilolite (BUC) (2 ewes). The clinoptilolite was characterized by Sheppard and Gude [3] as follows: 60% purity; 64.3% SiO₂; 12.2% Al₂O₃; ammonium ion binding capacity of 1.88 meq/g; particle size, -50 mesh. Ewes were penned individually in raised galvanized pens with expanded metal floors and were fed their respective diets *ad libitum* throughout the 5-day feeding period. New feed was added at 9 a.m. daily immediately after the 9 a.m. blood and rumen samples were obtained. Although no attempt was made to monitor pattern of daily feed intake, a large meal was generally consumed immediately after the addition of fresh feed. Water was available at all times from automatic nipple waterers. Two days before assignment to diets, jugular blood samples

and rumen fluid samples were obtained at 9 a.m. and at 3 p.m. for determination of pH (rumen fluid only), blood hematocrit, blood hemoglobin, and ammonia-N, urea-N, glucose, lactate, Ca, inorganic P, Mg, Na and K (Gilford Clinical Analyzer). One day after experimental diets were introduced, rumen fluid samples and jugular blood samples were obtained at 9 a.m. and 3 p.m. for the same measurements. The same procedure was followed on days 2, 3, 4 and 5. Final body weight of each ewe was recorded after the p.m. rumen fluid and blood samples were obtained on day 5. Feed consumed during the 5-day experimental diet feeding period was recorded for each ewe. All data on rumen fluid and blood traits were subjected to least-squares analysis of variance in a split plot design [20] with diet (animal) as whole plots, and day and time of day (period) as split plots. Simple correlations were calculated between selected pairs of traits.

RESULTS AND DISCUSSION

Body weight, feed intake and blood data are summarized in Table II and rumen fluid data are in Table III. Figures 1 through 9 show time trends of traits for which significant diet effects were recorded. Hematocrit (packed cell volume) and hemoglobin were affected by diet ($P < .01$). Hematocrit was consistently higher in ewes fed BU or BUC than in those fed B, indicating hemoconcentration. Although hematocrit of B

TABLE II. EFFECTS OF UREA OR UREA PLUS CLINOPTILOLITE SUPPLEMENTATION ON FEED INTAKE AND BLOOD TRATIS OF MATURE EWES FED A HIGH CONCENTRATE DIET^a

	Diet ^b			SD	Proba- bility
	Control	Urea	Urea + Clino		
No. of sheep	2	3	2		
Body wt., kg	74.6	61.4	79.2		
Daily feed, g	1377	995	1297		
Hematocrit (packed cell volume, %)	29.7	31.7	30.9	2.0	<.01
Hemoglobin, g/dl	10.5	11.4	11.3	0.7	<.01
Plasma ammonia N, mg/dl	.53	.94	1.14	.50	<.01
Plasma urea N, mg/dl	16.4	18.9	23.3	4.7	<.01
Plasma glucose, mg/dl	64.7	65.2	67.5	6.4	NS
Plasma lactate, mg/dl	15.1	12.3	12.3	6.4	NS
Plasma Ca, mg/dl	7.8	7.6	8.4	1.8	<.05
Plasma inorganic P, mg/dl	5.9	4.6	4.6	1.3	<.01
Plasma Mg, mg/dl	2.0	2.0	2.1	0.4	NS
Plasma Na, mg/dl	3642	3578	3663	355	NS
Plasma K, mg/dl	223	207	200	32	<.03

^a Each value is the mean of a total of 12 samples taken 2 days before diets were assigned (a.m. and p.m.) and on days 1, 2, 3, 4 and 5 (a.m. and p.m.) after diets were introduced.

^b Diets were fed ad libitum throughout the experiment. For diet composition see Table I.

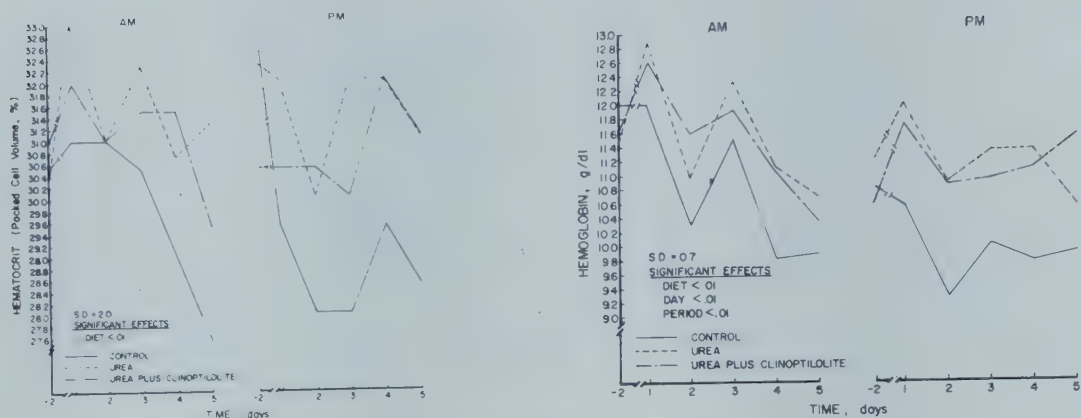


Figure 1. Effect of time and diet on blood hemoglobin (A) and hematocrit (B).

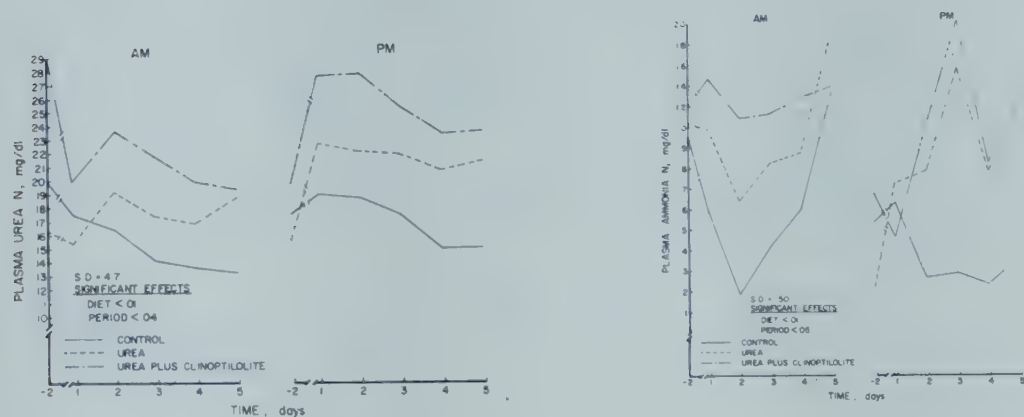


Figure 2. Effect of time and diet on plasma urea-N (A) and ammonia-N (B) concentrations.

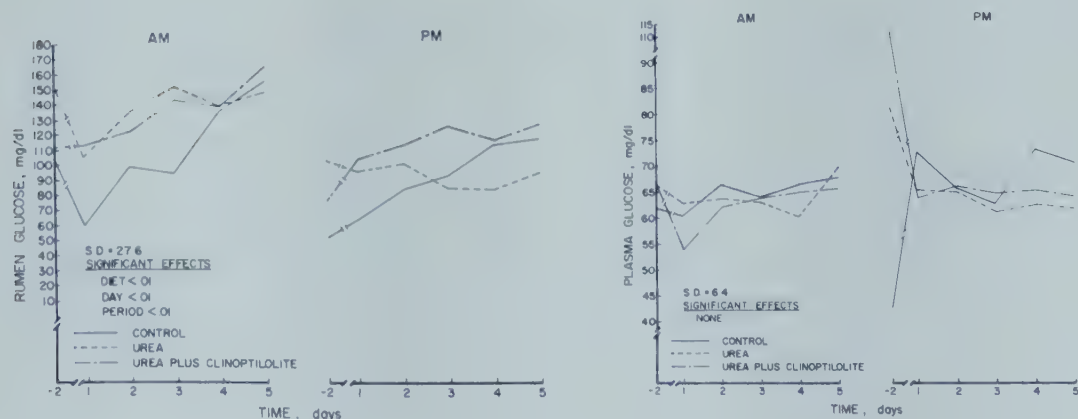


Figure 3. Effect of time and diet on plasma rumen glucose (A) and plasma glucose (B) concentrations.

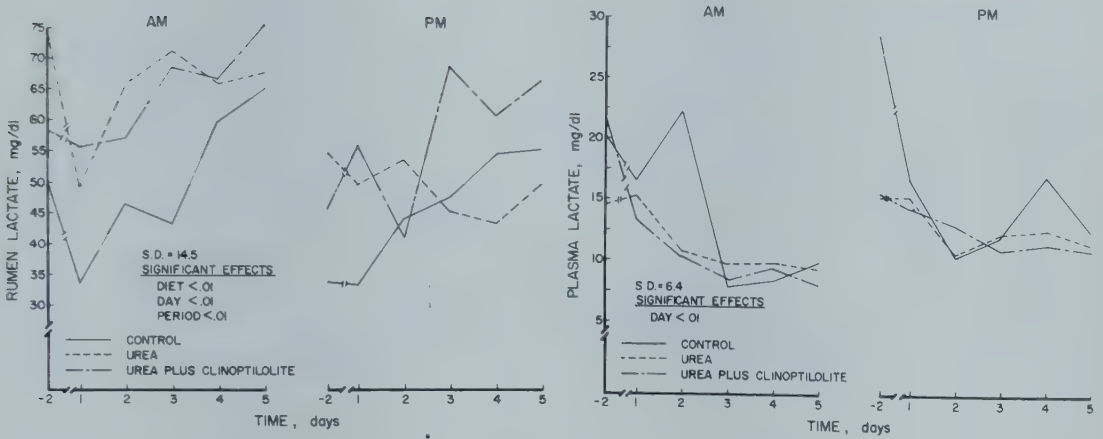


Figure 4. Effect of time and diet on plasma rumen lactate (A) and plasma lactate (B) concentrations.

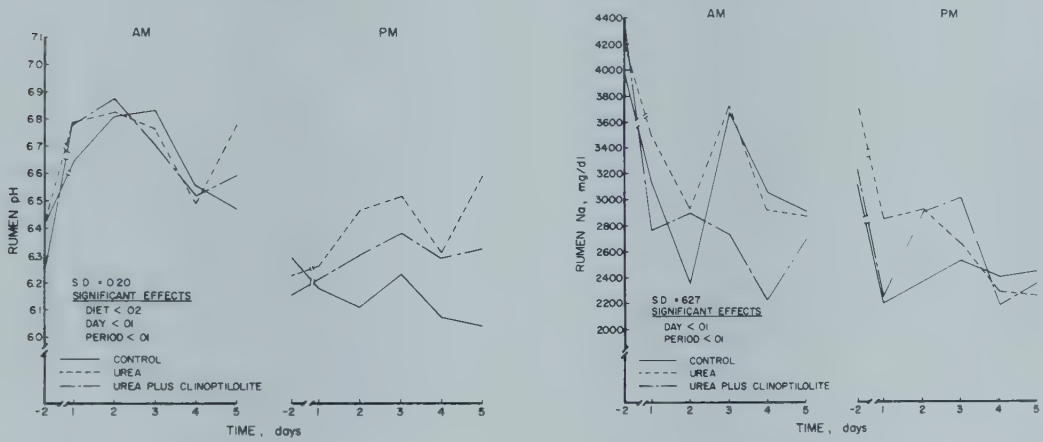


Figure 5. Effect of time and diet on plasma rumen pH (A) and rumen sodium (B) concentrations.

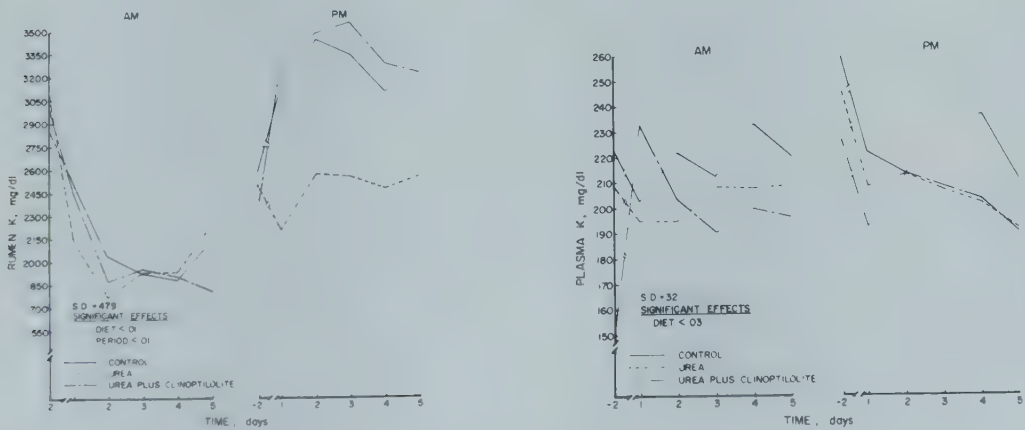


Figure 6. Effect of time and diet on plasma rumen K (A) and plasma K (B) concentrations.

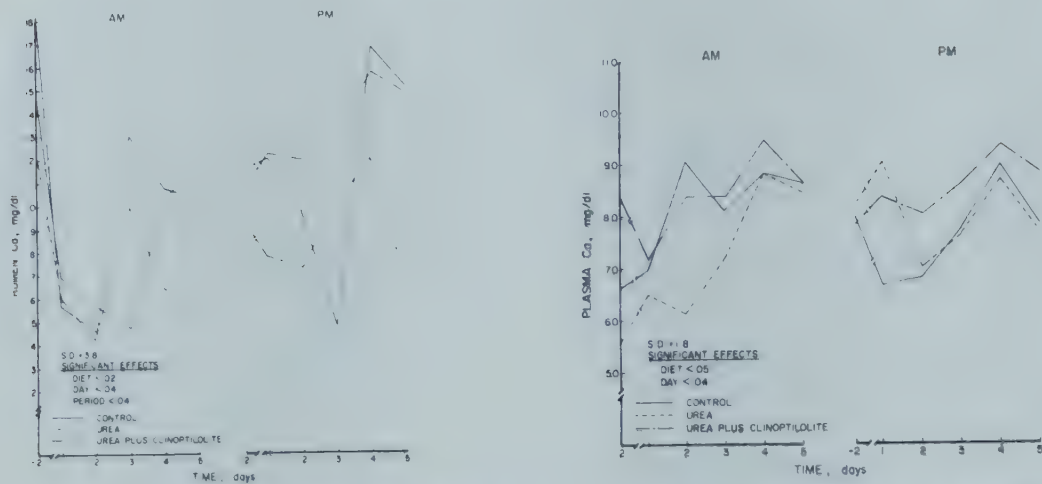


Figure 7. Effect of time and diet on plasma rumen Ca (A) and plasma Ca (B) concentrations.

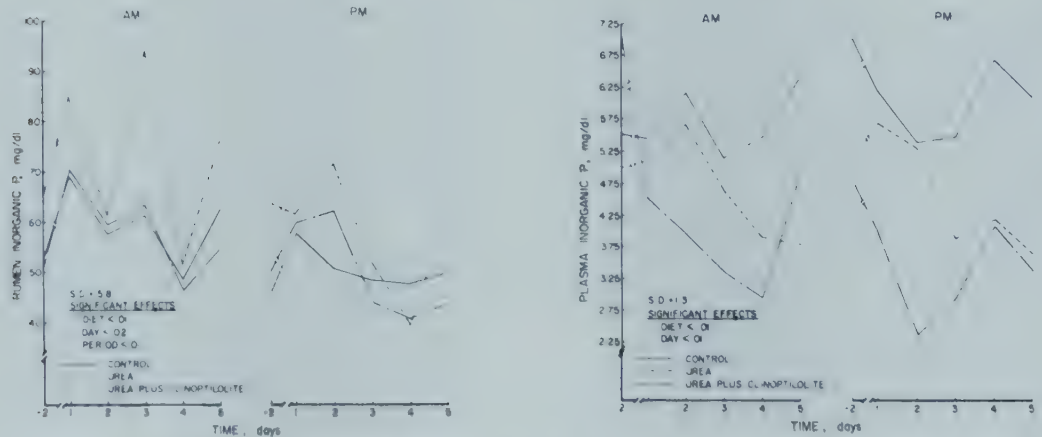


Figure 8. Effect of time and diet on plasma rumen inorganic P (A) and plasma inorganic P (B) concentrations.

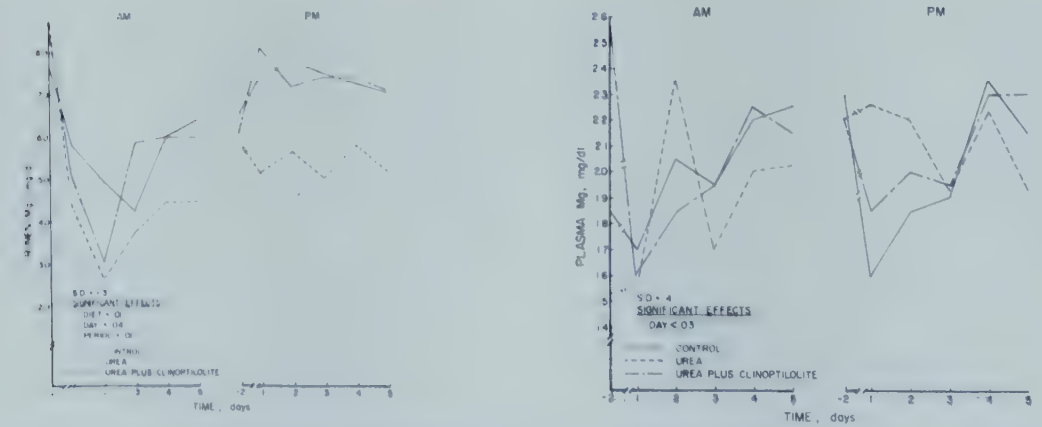


Figure 9. Effect of time and diet on plasma rumen Mg (A) and plasma Mg (B) concentrations.

ewes tended to decline with time, there was no significant effect of time (day) or of time of day (period) on it; hemoglobin was reduced ($P<.01$) with time in all groups and was higher ($P<.01$) in a.m. than in p.m. Apparent shifts in fluid movement in and out of the cardiovascular compartment may reflect changes in osmotic pressure and electrolyte metabolism in response to urea administration. Clinoptilolite did not appear to influence the response to urea with respect to either hematocrit or hemoglobin.

TABLE III. EFFECTS OF UREA OR UREA PLUS CLINOPTILOLITE SUPPLEMENTATION TO CONCENTRATE DIET ON RUMEN pH AND ON CONCENTRATIONS OF METABOLITES AND MINERAL IN RUMEN FLUID OF MATURE EWES^a

Trait	Diet ^b			SD	Probability
	Control	Urea	Urea + Clino		
No. of sheep	2	3	2		
Rumen pH	6.39	6.54	6.45	0.20	<.02
Glucose, mg/dl	96.8	115.6	120.7	27.6	<.01
Lactate, mg/dl	47.0	57.2	60.2	14.5	<.01
Ca, mg/dl	10.3	8.1	11.7	3.8	<.02
Inorganic P, mg/dl	54.2	65.3	54.0	15.8	<.01
Mg, mg/dl	6.5	4.9	6.5	1.3	<.01
Na, mg/dl	2850	3070	2800	627	NS
K, mg/dl	2677	2301	2678	479	<.01

^a Each value is the mean of a total of 12 samples taken 2 days before diets were assigned (a.m. and p.m.) and on days 1, 2, 3, 4 and 5 (a.m. and p.m.) after diets were introduced. Rumen urea and ammonia values were highly variable and are not reported. The variability may have been associated with faulty sampling technique.

^b Diets were fed ad libitum throughout the experiment. For diet compositions see Table I.

Rumen pH was increased in BU or BUC ewes compared with B ewes ($P<.02$). Samples taken in a.m. were higher ($P<.01$) in pH than those taken in p.m. and there appeared to be a general increase with time to day 2 with those taken on days 1 and 2 higher ($P<.01$) than those taken later. The large effect of time of day (period) on rumen pH appears to be an important consideration in studies of adaptation to diets containing urea.

Rumen urea-N concentration was increased ($P<.02$) by urea feeding. Concentration of plasma urea-N was higher ($P<.01$) in BUC than in BU ewes; B ewes had lower concentrations of plasma urea-N than ewes fed other diets at all periods (a.m. and p.m., except a.m. of day 1). There was no effect of day on plasma urea-N, but values for all diet groups were higher ($P<.04$) in p.m. than in a.m. Plasma ammonia-N was higher ($P<.01$) in BU and BUC ewes than in B ewes. Plasma concentrations were higher ($P<.05$) in p.m. than in a.m., with peak values occurring in the p.m. of day 3 for BU and BUC groups. Ammonia is absorbed through the ruminal wall at a faster rate when the rumen pH is high than when it is

low [21,22]. The relationship between rumen pH and plasma ammonia concentration in the present experiment fits with such an effect if one considers p.m. curves, but appears to be opposite of what would be expected when the a.m. curves are considered. The dynamics of rumen pH, rumen urea-N, rumen ammonia-N and plasma urea-N and ammonia-N interrelationships associated with feed consumption, enterohepatic circulation, salivary secretion and other physiological functions must be considered in the interpretation of this type of data.

Rumen glucose and lactate concentrations were lower ($P<.01$) in B than in BU and BUC ewes and increased ($P<.01$) steadily during the 5-day feeding time. Rumen glucose and lactate concentrations were higher ($P<.01$) in a.m. than in p.m. Neither plasma glucose nor lactate were affected by diet; plasma lactate declined ($P<.01$) during the 5-day feeding time. Failure of these two metabolites to increase during adaptation to a urea-containing diet indicates that plasma ammonia did not reach toxic concentrations, even though its peak concentration exceeded 1 mg/dl, which is more than twice that suggested as the toxic threshold [8].

Rumen concentrations of Ca ($P<.02$), Mg ($P<.01$), and K ($P<.01$) were decreased and that of inorganic P ($P<.01$) was increased by the BU diet; the presence of clinoptilolite (BUC) appeared to counteract these effects of urea, because overall means of B and BUC were similar for each mineral element. Rumen Ca tended to increase ($P<.04$) during the 5-day feeding period and to be higher ($P<.04$) in p.m. than in a.m. Effects of day on rumen fluid concentrations of Mg and K were less clear while inorganic P declined ($P<.02$) through time. Rumen fluid Mg and K concentrations were higher ($P<.01$) in p.m. than in a.m., while the opposite was true for inorganic P. There was no effect of diet on rumen fluid Na concentration, but the level declined ($P<.01$) over days and was higher ($P<.01$) in a.m. than in p.m. Plasma Ca was reduced ($P<.05$) by the BU diet; values for B and BUC groups tended to be similar except that values for B were considerably less than for those for BUC in the p.m. of days 1, 2 and 3. Plasma inorganic P concentration was reduced ($P<.01$) by urea and the reduction appeared to be more severe with the BUC diet than with BU, suggesting an exacerbating effect of clinoptilolite. Plasma Ca and inorganic P concentrations were affected ($P<.01$) by day of sampling, but not by time of day. Plasma Mg was not affected by diet but was affected ($P<.03$) by day of sampling, the biological importance of which is unclear. Plasma Na concentration was unaffected by diet, day or time of day. Plasma K concentration was reduced ($P<.03$) by urea in line with the reduced concentration of K in rumen fluid. Values for ewes fed the BUC diet tended to be lower than those for the BU diet at a.m. sampling times, suggesting that clinoptilolite may have bound K in accordance with its known K^+ exchange capacity. However, the trend was not consistent over days or time of day.

Simple correlations between traits are shown in Table IV. Only those pairs of traits shown to be significant at the $P<.01$ level of probability are reported. Cause-effect relationships may be operative in some, but not necessarily all, of the cases in which statistically significant correlations were recorded.

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TABLE IV. SIGNIFICANT ($<.01$) CORRELATIONS BETWEEN TRAITS (r Values)

Traits (84 observations per trait)

Rumen pH		Plasma urea N	
with:		with:	
Hemoglobin	.26	rumen urea N	.60
Plasma-		plasma ammonia N	.36
glucose	-.27	rumen Mg	.32
lactate	-.30	plasma Ca	.35
Mg	-.33	rumen Ca	.26
inorganic P	-.35	rumen inorganic P	-.39
		rumen K	.46
Rumen-		Plasma Mg	
glucose	.22	with:	
Mg	-.67	plasma Ca	.49
Ca	-.63	rumen Ca	.26
inorganic P	.54	plasma inorganic P	.39
K	-.58	rumen inorganic P	-.40
Rumen Ca		Hematocrit	
with:		with:	
rumen inorganic P	-.42	plasma lactate	.34
rumen K	.48	rumen lactate	-.31
		rumen Mg	-.30
Plasma Ca		Plasma ammonia N	
with:		with:	
rumen inorganic P	-.42	plasma inorg. P	-.26
plasma Na	-.28		
Plasma inorganic P		Rumen glucose	
with:		with:	
plasma lactate	.35	rumen lactate	.92
plasma Mg	.39	rumen K	-.27
Rumen K		Plasma lactate	
with:		with:	
rumen glucose	-.27	rumen Mg	.26
rumen Ca	.48	plasma inorg. P	.35
		plasma K	.29
Hemoglobin		Rumen Mg	
with:		with:	
hematocrit	.67	rumen Ca	.71
rumen inorganic P	.31	rumen inorg. P	-.40
rumen Na	.35	rumen K	.72

Rumen pH was positively correlated with rumen glucose ($r=.22$) and inorganic P ($r=.54$), but negatively correlated with plasma glucose ($r=-.27$), lactate ($r=-.30$), Mg ($r=-.33$) and inorganic P ($r=-.35$) and with rumen Mg ($r=-.67$), Ca ($r=-.63$) and K ($r=-.58$). Hematocrit and hemoglobin were highly correlated ($r=.67$); hemoglobin was correlated

with rumen inorganic P ($r=.31$) and rumen Na ($r=.35$), supporting the concept of osmotic shifts of electrolytes and fluids between blood and rumen fluid. Plasma urea-N was positively correlated with plasma ammonia-N ($r=.36$), and with plasma and rumen Ca ($r=.35$, $.26$), rumen Mg ($r=.32$) and rumen K ($r=.46$) and negatively correlated with rumen inorganic P ($r=-.39$). Plasma ammonia-N was correlated negatively with plasma inorganic P ($r=-.26$) which, in turn, was positively correlated with plasma lactate ($r=.35$) and Mg ($r=.39$).

Several significant correlations between plasma mineral concentrations (Mg with Ca, $r=.49$; Mg with inorganic P, $r=.39$) and between plasma mineral and plasma lactate (lactate with inorganic P, $r=.35$; lactate with K, $r=.29$) suggest important interactions among and between mineral elements and organic constituents related to rumen metabolism. Rumen glucose and lactate were highly correlated ($r=.92$). Similarly, rumen Mg was correlated positively with rumen Ca ($r=.71$), and rumen K ($r=.72$), but negatively with rumen inorganic P ($r=-.40$).

It is pertinent to note that these variations in rumen and plasma concentrations of metabolites and mineral elements and their numerous correlated changes associated with urea and urea plus clinoptilolite feeding, were induced in animals fed diets adequate and similar in concentrations of all known nutrients for adult sheep. Ludwick *et al.* [23,24] studied adaptation phenomena in lambs fed urea or soybean meal and reported higher ruminal NPN and ammonia-N and blood urea N in lambs fed urea, with a tendency for decreases with time. Source of dietary nitrogen did not significantly affect digestibility or retention of Ca, Mg or K [23]. Moore *et al.* [25] observed a decrease in Mg retention in lambs fed high versus low protein diets, but there were no differences due to form of nitrogen (intact protein or urea) in the diets. Ca retention was lower and P retention higher during the early part of the experiment in lambs fed the higher N diets; P retention was reduced in diets containing urea. Blood serum Ca, Mg, P and K concentrations were not affected by dietary nitrogen level or source. Adaptation of ruminants to urea feeding appears to be associated with adjustments in movement of fluids and electrolytes among body compartments and between blood and rumen lumen. Clinoptilolite, whose ammonia- and cation-binding properties appear operative at physiological pH, appears to modulate the response of the rumen and host animal to urea during early dietary adaptation. Voluntary daily feed intake during the first 5 days of adaptation to urea supplementation was greater when clinoptilolite was present in the diet than when it was absent (1377, 995 and 1297 g for ewes fed B, BU and BUC diets, respectively). This apparent difference may have been related more to ewe body weights than to diet. Unpublished observations (Pond, 1985) of feed intake patterns of 16 ewes fed urea with or without supplemental clinoptilolite failed to show feed intake depression following introduction to the diet containing urea. Ewes fed *ad libitum* a basal diet similar to that used in the present experiment or the same diet containing 1% urea alone, or in combination with 2 or 4% clinoptilolite consumed their diets at levels sufficient to sustain body weight gain of 2.6, 2.5, 2.6 and 1.3 kg, respectively, over a 16-day period. The full significance of and possible avenues for exploiting the effects of clinoptilolite on adaptation to high urea diets for ruminants need to be explored.

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LITERATURE CITED

1. NRC. Urea and other nonprotein nitrogen compounds in animal nutrition. National Academy of Sciences, Washington, DC, p 1-120 (1976).
2. Pond, W.G. and Mumpton, A.J. Zeo-Agriculture: Use of Natural Zeolites in Agriculture and Aquaculture. Westview Press, Boulder, CO, pp 1-296 (1984).
3. Sheppard, R.A. and Gude, A.J. 3rd. Mineralogy, chemistry, gas absorption and NH_4^+ -exchange capacity for selected zeolite tuffs from the Western United States. USDI Geological Survey, Open-File Report 82-969, p 1-15, (1982).
4. Visek, W.J. Some aspects of ammonia toxicity in animal cells. J. Dairy Sci. 51, 281 (1968).
5. Visek, W.J. Effects of urea hydrolysis on cell life-span and metabolism. Fed. Proc. 31, 1178 (1972).
6. Prior, R.L., Clifford, A.J., Gibson, G.E. and Visek, W.J. Effects of insulin on glucose metabolism in hyperammonium rats. Amer. J. Physiol. 221(2), 431 (1971).
7. Chow, Kye-Wing, Pond, W.G. and Walker, E.F., Jr. Blood metabolites in the hyperammonemic pig. Proc. Soc. Exp. Biol. Med. 134, 122 (1970).
8. Bartley, E.E., Avery, T.B., Nagaraja, T.G., Watt, B.R., Davidovich, A., Galitzer, S. and Lassman, B. Ammonia toxicity in cattle. V. Ammonia concentration of lymph and portal, carotid and jugular blood after the ingestion of urea. J. Anim. Sci. 53, 494 (1981).
9. Pond, W.G., Yen, J.T. and Hill, D.A. Decreased absorption of orally administered ammonia by clinoptilolite in rats. Proc. Soc. Exp. Biol. Med. 166, 369 (1981).
10. Pond, W.G. Protection against acute ammonia toxicity by clinoptilolite in mature sheep. Nutr. Rep. Internatl. 30, 991 (1984).

NUTRITION REPORTS INTERNATIONAL

11. Jacobi, V., Vrzgula, L., Blazovsky, J., Havassy, I., Ledecy, V. and Bartko, P. Study of the influence of zeolite (clinoptilolite) on the dynamics of some parameters of N-metabolism after feeding in v. portae, v. jugularis and rumen fluid in bulls. *Veter. Med. (Praha)* 29(4), 207 (1984).
12. Repp, W.W., Hale, W., Chang, E.W. and Burroughs, W. The influence of and administration of nonprotein nitrogen feeding compounds upon blood ammonia and urea levels in lambs. *J. Anim. Sci.* 14, 118 (1955).
13. Word, J.D., Martin, L.C., Williams, D.L., Williams, E.I., Panciera, R.J., Nelson, T.E. and Tillman, A.D. Urea toxicity studies in the bovine. *J. Anim. Sci.* 29, 786 (1969).
14. Barej, W. The influence of ammonia ion on carbohydrate and protein metabolism in the liver of rat and sheep. *Warsaw Agric. Univ. Final Report PL-ARS-77, Warsaw, Poland, p 1-96* (1983).
15. Adams, D.C. and Kartchner, R.J. Effect of level of forage intake on rumen ammonia, pH, liquid volume and liquid dilution rate in beef cattle. *J. Anim. Sci.* 58, 708 (1984).
16. Chalupa, W. Metabolic aspects of nonprotein nitrogen utilization in ruminant animals. *Fed. Proc.* 31, 1152 (1972).
17. Hemken, R.W., Harmon, R.J. and Mann, L.M. Effect of clinoptilolite on lactating dairy cows fed a diet containing urea as a source of protein. In: *Zeo Agriculture: Use of Natural Zeolites in Agriculture and Aquaculture*, (W.G. Pond and F.A. Mumpton, editors). Westview Press, Boulder, CO. p 171-176, (1984).
18. Sweeney, T.F., Cervantes, A., Bull, L.S. and Hemken, R.W. Effect of dietary clinoptilolite on digestion and rumen fermentation in steers. In: *Zeo Agriculture: Use of Natural Zeolites in Agriculture and Aquaculture*, (W.G. Pond and F.A. Mumpton, editors). Westview Press, Boulder, CO. p 177-187 (1984).
19. Pond, W.G., Laurent, S.M. and Orloff, H.D. Effect of dietary clinoptilolite or zeolite Na-A on body weight gain and feed utilization of growing lambs fed urea or intact protein as a nitrogen supplement. *Zeolite* 4, 127 (1984).
20. SAS. Statistical Analysis Systems, Inc., Cary, NC. (1979).
21. Coombe, J.B., Tribe, D.E. and Morrison, J.W.C. Some experimental observations on the toxicity of urea to sheep. *Aust. J. Agric. Res.* 11, 247 (1960).
22. Hogan, J.P. The absorption of ammonia through the rumen of sheep. *Aust. J. Biol. Sci.* 14, 448 (1961).
23. Ludwick, R.L., Fontenot, J.P. and Tucker, R.E. Studies of the adaptation phenomenon by lambs fed urea as the sole nitrogen

source: digestibility and nutrient balance. J. Anim. Sci. 33, 1298 (1971).

24. Ludwick, R.L., Fontenot, J.P. and Tucker, R.E. Studies of the adaptation phenomenon by lambs fed urea as the sole nitrogen source: chemical alterations in ruminal and blood parameters. J. Anim. Sci. 35, 1036 (1972).
25. Moore, W.F., Fontenot, J.P. and Webb, K.E., Jr. Effect of form and level of nitrogen on magnesium utilization. J. Anim. Sci. 35, 1046 (1972).

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CONCENTRATION OF ZINC AND COPPER IN SMALL SAMPLES OF FORE- AND HINDMILK

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ABSTRACT

Eighty-seven samples of small amounts (~5 ml) of human fore- and hindmilk were studied at different stages of lactation (2-12 weeks). There were no significant differences between fore- and hindmilk for zinc or copper measured as mass/volume or in relation to total mineral content. The concentration of zinc and copper in all samples showed very low ($r = 0.05$) and non-significant correlation coefficients. The findings confirm earlier work on larger samples and the discussion includes recommendation for sampling human milk.

INTRODUCTION

The variability in the occurrence of nutritional constituents in human milk is of importance in ascertaining the concentration of the chosen nutrient as the true value or its best representation. As opposed to studies in experimental animals where whole discharges of mammary gland are obtained, thus creating the ideal sampling conditions, in human field studies a small sample is desirable because it interferes the least with nursing and leaves the majority of the milk for the child. A sampling technique taking into consideration variable major milk constituents has been studied (1) and proposed as representative of a feeding. A similar method was independently adopted in our studies (2). Although the variability of human constituents has been known a long time, only major constituent have been adequately investigated. As for trace elements, very few reports exist exploring their variability during a feeding. For iron, a significant difference between fore- and hindmilk has been found (3). Both the significant correlation of iron with milk fat (4) and the finding that 31% of total iron in human milk is associated with fat (5) seems to confirm the difference between fore- and hindmilk. Although the occurrence of zinc and copper has been reported in the order of 12 and 15 percent of their total content in milk (5), both Picciano (3) and Neville et al. (6) suggest no significant differences between fore- and hindmilk.

While Picciano (3) took 15 ml for each fore- or hindmilk sample of a single nursing, Neville et al (6) defined mid- and hind samples of milk in terms of minutes

after feeding began. During our study, we sampled small amounts of milk immediately before and after the feeding of the child, and thus were able to study the presence of zinc and copper in samples more representative of fore- and hindmilk and over a longer period of lactation than Picciano (3) and Neville et al. (6).

MATERIALS AND METHODS

As part of a larger project, the detailed description of the subjects participating in this study has been published elsewhere (2) and briefly will be mentioned here. Milk samples of approximately 5 ml were obtained by manual expression from 13 mothers at different stages of lactation, before and after the feeding of the child. Precautions against contamination of zinc and copper were taken by using deionized water and rendering the glassware metal-free, according to the protocol adopted by our laboratory. After glassware was cleaned, it was rinsed consecutively in nitric acid, EDTA, and deionized water. Samples were chilled and taken to the laboratory and kept under temperatures of -20°C until time of analyses. To measure zinc and copper, aliquots of samples of whole milk were dried at 105°C overnight, carbonized at 250°C for 4-5 hours and ashed in a muffle furnace with the aid of 0.1 ml of concentrated nitric acid (Suprapur-Merck, Darmstadt, West Germany) according to the procedure described by Vuori and Kuitunen (7). Determination of zinc and copper was done by atomic absorption spectrophotometry. The method was calibrated by using reference solutions of standards (Normex-Carlo Erba, Milano, Italy). Lipid and ash were determined by A.O.A.C. methods as described (2). Results were reported as mass/volume and as mass/mass of ash on a fat-free basis.

RESULTS AND DISCUSSION

The results presented in Table 1 show non-significant differences in mean values between fore- and hindmilk for either zinc or copper. Only when total mineral content of milk was taken into consideration the difference between fore- and hindmilk for zinc approached significance ($P < 0.07$). These same samples also showed non-significant differences in ash between fore- and hindmilk. The correlation between zinc and copper in human milk in either fore- and hindmilk was very low ($r = 0.05$) and not significant*.

Since the samples in this study comprised stages of lactation ranging from 2 to 12 weeks, it may be argued that at least for zinc, the likely decrease in concentration observed during the first three months (7) can be larger than overall differences between fore- and hindmilk. As observed by Vuori and Kuitunen (7) the median concentration of zinc

TABLE 1 - CONCENTRATION OF ZINC AND COPPER IN FORE- AND HINDMILK

	Foremilk (46) ¹	Hindmilk (41)	P
Zinc			
μg/ml	1.18 ± 0.94 ²	1.13 ± 1.17	N.S.
mg/g ash ³	0.70 ± 0.49	0.79 ± 0.74	N.S.
Copper			
μg/ml	0.78 ± 0.20	0.78 ± 0.25	N.S.
mg/g ash ³	0.49 ± 0.19	0.58 ± 0.29	N.S.
Total ash			
g/100 ml	0.17 ± .04	0.16 ± 0.06	N.S.

¹ Number of observations

² Mean ± SD

³ Fat-free basis

can fall to 25% between the first two and twelve weeks of lactation. Therefore, further statistical analysis of the zinc (mass/volume) was selectively applied to 17 samples at the first month (1.93 ± 0.28 for foremilk; 2.08 ± 0.43 for hindmilk) and to 16 samples at the third month (0.75 ± 0.11 for foremilk; 0.78 ± 0.15 for hindmilk) and no statistically significant differences were found between fore- and hindmilk in neither the first nor in the third month of lactation.

Our results for zinc and copper are in agreement with several reports. Compared to Picciano (3), our values for zinc are comparable whereas for copper our values are higher and in the upper range of the literature. Also in agreement with Munch-Petersen (8) and Picciano (3) are our findings of no significant differences between fore- and hindmilk for zinc and copper. The differences between fore- and hindmilk for ash were not significant. Although as previously reported by Macy *et al* (9) the concentration of ash in human milk does not remain constant but also does not appear to undergo fixed variations. This is also a conclusion that can be extended specifically for zinc and copper in fore- and hindmilk. In the same study, Macy *et al* (9) have suggested an intimate relationship between many pairs of constituents of human milk. Thus one could speculate that the rate of secretion of one would change according to the other. We tested this association for zinc and copper and found no relationship in the total content secreted either in fore- or in hindmilk.

Although Picciano (3) has suggested that a one single sample from a feeding would supply a representative

estimate, we suggest that given the great variability found in these minerals during the feeding at least two samples should be secured from an individual. Due to variation in the total volume of milk secreted during a feeding (which we did not study), we also suggest that the concentration of individual minerals be analyzed with respect to total mineral content in order to standardize the measure.

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REFERENCES

1. Prentice, A., Prentice, A.M. and Whitehead, R.G. Breast milk fat concentrations of rural African women. 1. Short term variations within individuals. *Br. J. Nutr.* 45, 483 (1981).
2. Dorea, J.G., Horner, M.R., Bezerra, V.L.V.A. and Campanate, M.L. Longitudinal study of major milk constituents from two different socioeconomic groups of mothers in Brazil. *Nutr. Rep. Intern.* 29, 699 (1984).
3. Picciano, M.R. Mineral content of human milk during a single nursing. *Nutr. Rep. Intern.* 18, 5 (1978).
4. Fransson, G-B. and Lonnerdal, B. Iron in human milk. *J. Ped.* 96, 380 (1980).
5. Fransson, G-B. and Lonnerdal, B. Zinc, copper, calcium, and magnesium in human milk fat. *Am. J. Clin. Nutr.* 39, 185 (1984).
6. Neville, M.C., Casey, C., Archer, P., Seacat, J. and Keller, R. Within feed and diurnal variation in selected nutrients in human milk. *Fed. Proc.* 42: 921, (1983).
7. Vuori, E. and Kuitunen, P. The concentration of copper and zinc in human milk, a longitudinal study. *Acta Paediat. Scand.* 68, 33 (1979).
8. Munch-Peterson, S. On the copper content in mother's milk before and after copper administration. *Acta Paediat. Scand.* 34, 378 (1950).
9. Macy, I.G., Nims, B., Brown, M. and Hunscher, H.A. Human milk studies. VII. Chemical analysis of milk representative of the entire first and last halves of the nursing period. *Am. J. Dis. Child.* 42, 569 (1931).

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PULMONARY FUNCTION AND TREADMILL PERFORMANCE OF MALES RECEIVING ASCORBIC ACID SUPPLEMENTS

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ABSTRACT

Cigarette smoking and nonsmoking untrained adult males performed pulmonary function and submaximal treadmill exercise tests over two periods of 6 wk duration while taking either 1000 mg ascorbic acid or placebo tablets daily in a crossover, double-blind study. The periods were separated by 4 wk. No differences between ascorbic acid and placebo treatments of smokers and nonsmokers were observed for 1-second forced expiratory volume, forced vital capacity, resting and post-exercise heart rates and diastolic and systolic blood pressures, treadmill workload, ventilation, and oxygen consumption. Nonsmokers had significantly lower and smokers tended to have lower differences in post-exercise blood lactic acid values after 3 wk ascorbic acid treatment as compared to 3 wk placebo; these differences were not significant after 6 wk. Ascorbic acid supplementation of 1000 mg daily for 3 to 6 wk tended to have little effect on the pulmonary function or treadmill performance of healthy smoking and nonsmoking males.

INTRODUCTION

Athletic performance is known to be impaired by vitamin C deficiency; when the deficiency is corrected via supplementation, physiological adjustment to exercise is enhanced. Some researchers (1-5) have reported that supplemental vitamin C increased athletic performance as ascertained by varying parameters. Other investigators have found athletic performance not to be affected by supplemental vitamin C (6-12). These researchers used varying levels of supplementation; in addition, many times the initial vitamin C status of the subjects was not determined prior to supplementation.

Cigarette smoking can cause temporary or acute changes in cardiovascular performance and pulmonary function (13-16). The effects of chronic cigarette smoking upon heart and lung functions are less clear in that researchers have reported mixed results (17-20). Differences between smokers and nonsmokers in response to treadmill exercise (20) as well as no differences (6) have been reported. Several researchers found that plasma and leukocyte levels of ascorbic acid are lower in cigarette smokers as compared to nonsmokers (20-23); however, reports in this area are not in total agreement (1,6).

Prior research in our laboratory (20) indicated that supplementary vitamin C at 300 mg daily for 3 wk had little effect on pulmonary function and treadmill performance of healthy smoking and nonsmoking males. The study was double-blind and subjects served as their own control,

taking either ascorbic acid or placebo tablets during different phases of the study. The vitamin C status and intakes of the subjects were determined before the initiation of ascorbic acid or placebo treatment.

In the present investigation pulmonary function and treadmill exercise measurements of healthy untrained male cigarette smokers and non-smokers were determined initially and during two periods of 6 wk duration each while consuming tablets containing either 1000 mg ascorbic acid or placebo daily; a random double-blind design was utilized. Before treatment, all subjects had normal vitamin C intakes and normal plasma vitamin C levels.

METHODS

Subjects

Cigarette smoking and nonsmoking (never smoked) healthy men, 23 to 40 yr of age, volunteered as subjects. A smoker was defined as an individual who had smoked cigarettes for at least 10 pack yr (number of packs daily multiplied by number of yr smoked). Both groups of subjects were untrained in that they did not participate in any regular exercise. Two of the smokers that received ascorbic acid during the first period did not participate during the third period and hence did not receive the placebo. Data obtained from these two subjects were included in the pretreatment (base-line) measurements but not in the analyses after treatment. The project was approved by the University's Review Board for Research Involving Human Subjects.

Design

The smoking and nonsmoking subjects were randomly divided into two groups. The experimental protocol was divided into three periods. During the first 6 wk period half of the subjects in each group were given two tablets daily containing 500 mg ascorbic acid (sodium) each and the other half received citric acid (sodium) tablets. These tablets, prepared by Hoffmann-LaRoche (Nutley, NJ), were given in a double-blind manner. All parameters were measured initially, after 3 wk, and after 6 wk.

After 6 wk the volunteers were taken off the tablets for 4 wk. During this second period none of the tests was performed; this period was included so that the tissues of the subjects that took ascorbic acid tablets during the first period could be desaturated. The half-life of ascorbic acid in humans is reported to be about 15 days (24).

During the third period (6 wk in length) subjects received the tablets not given during the first period. Parameters were measured at the beginning of the third period, after 3 wk, and after 6 wk. Two of the smokers who received ascorbic acid during the first period did not participate during the final period and hence did not receive the placebo.

Pretreatment

Information regarding smoking habits and age was obtained from each subject. A three-day food record (25) was obtained from each subject and

the vitamin C intakes of the subjects were calculated using food composition tables (26); subjects consuming more than 150 mg of the vitamin daily were excluded.

Height; weight; plasma vitamin C levels; 1-second forced expiratory volume, FEV₁; forced vital capacity, FVC; the FEV₁/FVC ratio, FEV%; resting and post-exercise blood pressure (BP) and heart rate (HR); treadmill workload, METS; inspired ventilation, \dot{V}_I ; oxygen consumption, $\dot{V}O_2$; and post-exercise blood lactic acid levels of the subjects were measured before each experimental period began. The exercise regimen consisted of subjects walking on a treadmill at 3.9 km/h and 0% grade for 3 min with the speed then being raised to 4.7 km/h for 5 min with the percentage grade being increased to a level that elicited a steady-level HR estimated to represent 50% of the predicted exercise HR reserve. Treadmill and ventilation measurements were performed during the last minute of the 5 min exercise period. On completion of the 5-min exercise, the treadmill speed was returned to 3.9 km/h and 0% grade for 3 min; at this time post-exercise measurements were performed. These measurements have been previously described (20). All smokers were requested not to smoke for at least 30 min before testing.

Statistical Analyses

Data obtained were evaluated using analysis of variance procedures (27); these procedures were also utilized for analyzing differences within and between groups for the various parameters during ascorbic acid and placebo treatments. Means (\bar{X}) and standard deviations (SD) were also calculated.

RESULTS AND DISCUSSION

Subject Description

Both groups of subjects had similar ages, heights, and weights; these were as follows: ($\bar{X} \pm \text{SD}$) smokers -- 29.9 \pm 5.4 yr, 175.3 \pm 4.8 cm, 73.4 \pm 7.6 kg and nonsmokers -- 30.5 \pm 4.5 yr, 176.7 \pm 5.2 cm, 70.8 \pm 7.5 kg. The cigarette smoking habit reported by the smokers was 15.7 \pm 10.0 pack-yr ($\bar{X} \pm \text{SD}$). The daily vitamin C intakes of the smokers were somewhat lower ($p < 0.14$) than those of nonsmokers; smokers reported daily consumption of 86.3 \pm 12.8 mg ($\bar{X} \pm \text{SD}$) and nonsmokers, 98.8 \pm 26.1 mg. These dietary intakes of the vitamin were generally maintained through the study. Other investigators (20-23) have reported finding lower plasma vitamin C levels in smokers than in nonsmokers.

Pretreatment Measurements

The pretreatment pulmonary function and treadmill performance values of smoking and nonsmoking subjects are given in Table I. Smokers had significantly lower ($p < 0.05$) FEV₁ and FVC values than nonsmokers. Non-significantly lower FEV₁ and FVC values had been observed previously for smokers as compared to nonsmokers (20). Marcq and Minette (28) reported significantly lower FEV₁ values in smokers than in nonsmokers. The FVC measurements observed in the present study are similar to those reported by Chevalier et al. (17) and Pelletier (23); however, Krumholz et al.

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Table I. Pretreatment Pulmonary Function and Treadmill Performance Values of Smoking and Nonsmoking Subjects

Measurement	Smokers	Nonsmokers
FEV ₁ (l)	3.0 ^a + 0.7	3.6 + 0.5
FVC (l)	4.0 ^a + 0.6	4.8 + 0.6
FEV %	76.5 + 11.9	77.6 + 5.9
Resting HR (beats/min)	72.1 + 12.2	66.9 + 10.9
Post-exercise HR (beats/min)	92.1 + 15.1	78.8 + 12.5
Resting Systolic BP (mm Hg)	117.1 + 9.9	122.1 + 14.2
Resting Diastolic BP (mm Hg)	73.9 + 8.3	73.5 + 6.5
Post-exercise Systolic BP (mm Hg)	131.7 + 14.3	129.0 + 13.3
Post-exercise Diastolic BP (mm Hg)	74.6 + 9.0	73.4 + 4.8
Treadmill Workload (METS)	5.4 ^a + 1.4	6.9 + 1.0
\dot{V}_I (l/min)	34.3 + 6.7	36.4 + 5.5
$\dot{V}O_2$ (ml/kg BW/min)	20.9 + 5.9	24.9 + 3.8
Post-exercise Blood Lactic Acid (mg/dl)	18.4 + 2.2	17.3 + 1.6
n	7	10

Values represent $\bar{X} \pm SD$.

^aSignificantly lower ($p < 0.05$) than values of nonsmokers.

(19) indicated that smokers had significantly lower FVC values than nonsmokers. The FEV% of smokers in the current study were nonsignificantly lower ($p < 0.25$) than those of smokers. Previous researchers in our laboratory had observed this difference to be significant at $p < 0.05$. Other investigators (17,28) have reported similar FEV% values. Lung function seems to be somewhat impaired in smokers as compared to nonsmokers.

The resting and post-exercise HR of smokers in the current study were nonsignificantly higher ($p < 0.46$ and < 0.07 , respectively) than those of nonsmokers. Resting and post-exercise diastolic and systolic BP measurements of smokers and nonsmokers were similar. HR and BP measurements of both groups were similar to reported values (18,20). Previous investigators (17-19) have reported higher resting HR values among cigarette smokers than nonsmokers.

Treadmill workload values of smokers in the current study were significantly lower ($p < 0.05$) than those of nonsmokers; this finding is in agreement with our previous observations (20). Nonsmokers seem to have a higher level of mechanical efficiency on the treadmill than smokers. No significant differences between smokers and nonsmokers were observed for \dot{V}_I , $\dot{V}O_2$, and post-exercise blood lactic acid measurements; this is in agreement with previous findings in our laboratory (20). Other researchers have reported smokers and nonsmokers to have similar \dot{V}_I and $\dot{V}O_2$ values (6,17,19) although increased O_2 debts have been observed in

smokers (17,19).

Smokers had significantly lower FEV₁, FVC, and treadmill workloads than nonsmokers. Smokers also had nonsignificantly lower plasma vitamin C and FEV% values and nonsignificantly higher resting and post-exercise HR measurements than nonsmokers. These trends continued through the study. All differences observed to be significant were also significant at the same levels when data from the five (rather than the seven) smokers that completed the study were compared to values of nonsmokers. Pre-treatment data were obtained at the beginning of the study but are not exactly the same as before ascorbic acid or before placebo values in that

Table II. Plasma Vitamin C Levels of Smokers and Nonsmokers Taking Ascorbic Acid and Placebo Tablets

Group	Treatment				Differences Between Changes During the 2 Treatments	
	Before	3 wk	6 wk	Placebo ^a	3 wk	6 wk
mg/dl						
Smokers	0.79 ±.13	1.00 ^b ±.16	1.30 ^c ±.18	0.82 ±.08	0.16 ^d ±.12	0.47 ^e ±.22
Nonsmokers	0.84 ±.12	1.33 ^c ±.20	1.37 ^c ±.20	0.88 ±.10	0.47 ^e ±.22	0.52 ^e ±.25

Values represent $\bar{X} \pm \text{SD}$.

^aIn that no significant differences were observed between data obtained before as well as after 3 and 6 wk of placebo administration, these values were combined.

^bValues approached being significantly higher ($p < 0.10$) than before ascorbic acid values.

^cSignificantly different ($p < 0.001$) from values obtained before ascorbic acid treatment and placebo measurements.

^dChanges during ascorbic acid treatment significantly different ($p < 0.05$) than changes during placebo.

^eChanges during ascorbic acid treatment significantly different ($p < 0.005$) than changes during placebo.

some subjects were first given ascorbic acid tablets while others received the placebo first.

Plasma Vitamin C Measurements

The plasma vitamin C levels of smokers and nonsmokers taking ascorbic acid and placebo tablets are given in Table II. Plasma vitamin C levels of smokers and nonsmokers increased significantly or almost so after 3 wk ($p < 0.10$ and 0.001 , respectively) and after 6 wk ($p < 0.001$) of ascorbic acid treatment as compared to values obtained before ascorbic

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acid treatment. As expected, plasma ascorbic acid levels of subjects after 3 or 6 wk of placebo treatment were similar to values obtained before the placebo treatment. Changes observed during the ascorbic acid treatment were significantly higher ($p < 0.05$ or 0.005) than changes during the placebo. This may be taken to indicate that subjects did take the vitamin C tablets. Similar data were observed in our laboratory previously (12). The values of both the smokers and nonsmokers after the ascorbic acid treatment indicated that the plasma was saturated with the vitamin as defined by Friedman et al. (29).

Spirometric Measurements

All three measures of pulmonary function in the smokers and nonsmokers before as well as after 3 and 6 wk of taking ascorbic acid tablets were similar to initial values and to those after 3 and 6 wk of placebo. Snigur (30) observed that the FVC values of children consuming 100 mg vitamin C daily for 2 yr were similar to their pretreatment values.

Cardiovascular Measurements

Resting and post-exercise HR as well as diastolic and systolic BP of smokers and nonsmokers were not significantly affected by the ascorbic acid treatment; these values were similar to initial measurements. Similar data were observed previously (12) when males received 300 mg ascorbic acid daily for 3 wk. Other researchers (5,8,9) have also found no differences in resting HR when subjects took vitamin C supplements. Harper et al. (31) reported that vitamin C (50 mg daily taken along with other vitamins) increased resting HR while other investigators (2,4,32) found that the supplementation decreased the resting or exercising HR. Kirchhoff (9) and Hoitink (2) both reported no changes in diastolic BP with administration of vitamin C. Kirchhoff (9) also observed no differences in systolic BP between subjects taking vitamin C and those on placebo. However, Hoitink (2) found that vitamin C supplementation resulted in a reduction in the systolic BP of his subjects.

Exercise Respiratory Measurements

No significant differences were observed in the MET equivalent attained in the treadmill exercise or in the \dot{V}_I , or $\dot{V}O_2$ measurements of smokers or nonsmokers between ascorbic acid and placebo treatments. Previous studies (12) indicated differences ($p < 0.05$) in $\dot{V}O_2$ values of nonsmokers between 300 mg daily ascorbic acid and placebo treatments.

The post-exercise blood lactic acid values for both groups obtained after 3 or 6 wk of ascorbic acid treatment were statistically similar to values before ascorbate (Table III). However, the post-exercise blood lactic acid values of nonsmokers obtained after 3 wk on ascorbic acid treatment were significantly lower ($p < 0.05$) than values after 3 wk on placebo. However, the differences between changes after 3 wk of ascorbic acid treatment compared to the differences after 3 wk of placebo were significant ($p < 0.01$) for nonsmokers and approached significance ($p < 0.10$) for smokers; these differences were nonsignificant after 6 wk. Thus, the ascorbic acid supplements may have been of some benefit after 3 wk but not after 6. Decreases in blood lactic acid are indicative of the workloads of individuals becoming more aerobic.

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Table III. Post-exercise Blood Lactic Acid Levels of Smokers and Nonsmokers Taking Ascorbic Acid and Placebo Tablets

Group	Treatment				Differences Between Changes During the the 2 Treatments	
	Ascorbic Acid			Placebo ^a	3 wk	6 wk
	Before	3 wk	6 wk			
	mg/dl					
Smokers	19.1 <u>+2.1</u>	18.4 <u>+2.3</u>	17.2 <u>+1.5</u>	19.0 <u>+4.1</u>	-0.7 <u>+1.8</u>	-1.8 <u>+1.5</u>
Nonsmokers	17.7 <u>+1.8</u>	16.4 ^b <u>+1.8</u>	17.3 <u>+2.1</u>	18.3 ^b <u>+1.8</u>	-1.4 ^c <u>+1.7</u>	-0.7 <u>+3.0</u>

Values represent $\bar{X} \pm \text{SD}$.

^aIn that no significant differences were observed between data obtained before as well as after 3 and 6 wk of placebo administration, these values were combined.

^bSignificantly different ($p < 0.05$) from each other.

^cChanges during ascorbic acid treatment significantly different ($p < 0.01$) than changes during placebo.

These findings with relation to lactic acid responses were similar to those reported previously from our laboratory in which smokers and nonsmokers received 300 mg ascorbic acid daily for 3 wk (12). However, Hoitink (2) and Keys and Henschel (8) found no differences in blood lactic acid levels with the addition of ascorbic acid to the diet. Lower lactic acid production has been observed when chick tibias were cultured for 5 days in a medium containing ascorbic acid than in medium lacking the vitamin (33). Thus, ascorbic acid seems to suppress blood lactic acid production. Nitzescu et al. (34) gave ascorbic acid to 8 patients with cardiac insufficiency and found that their blood lactic acid levels fell following gentle exercise; lactic acid levels increased after exertion but less when the vitamin was given. Vitamin C may function in the stimulation of skeletal aerobic metabolism.

Differences were more evident between smokers and nonsmokers than between the ascorbic acid and placebo treatments. Each subject in this study served as his own control in that he was submitted to both the ascorbic acid and the placebo treatments. Considerable individual variation was observed in all the parameters; parameter values taken on the same individuals also varied to some degree. Supplementary vitamin C at 1000 mg daily for 3 and 6 wk seemed to have little effect on pulmonary function and treadmill performance of healthy smoking and nonsmoking males except for post-exercise blood lactic acid measurements.

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REFERENCES

1. Yeung, D.L. Relationships between Cigarette Smoking, Oral Contraceptives, and Plasma Triglycerides and Cholesterol. *Am. J. Clin. Nutr.* 29, 1216 (1976).
2. Hoitink, A.W.J.H. Vitamin C en Arbeid: Onderzoekingen over den Invloed van Arbeid en van Vitamin C-toediening op het Menschelijk Organisme. *Verh. Nederlands Inst. Praevent. Geneesk.* 4, 1 (1946).
3. Hoogerwerf, A. and Hoitink, A.W.J.H. The Influence of Vitamin C Administration on the Mechanical Efficiency of the Human Organism. *Int. Z. Angew. Physiol. Einschl. Arbeitsphysiol.* 20, 164 (1963).
4. Howald, H., Segesser, B. and Korner, W.F. Ascorbic Acid and Athletic Performance. *Ann. N. Y. Acad. Sci.* 258, 458 (1975).
5. Meyer, B.J., de Bruin, E.J.P., Brown, J.M.M., Bieler, E.U., Meyer, A.C. and Grey, P.C. The Effect of a Predominantly Fruit Diet on Athletic Performance. *Plant Foods Man* 1, 233 (1975).
6. Bailey, D.A., Carron, A.V., Teece, R.G. and Wehner, H.J. Vitamin C Supplementation related to Physiological Response to Exercise in Smoking and Nonsmoking Subjects. *Am. J. Clin. Nutr.* 23, 905 (1970).
7. Bender, A.E. and Nash, A.H. Vitamin C and Physical Performance. *Plant Foods Man* 1, 217 (1975).
8. Keys, A. and Henschel, A.F. Vitamin Supplementation of U.S. Army Rations in Relation to Fatigue and the Ability to do Muscular Work. *J. Nutr.* 23, 259 (1970).
9. Kirchhoff, H.W. Über den Einfluss von Vitamin C auf Energieverbrauch, Kreislauf- und Ventilationsgrößen im Belastungsversuch. *Nutr. Dieta* 11, 184 (1969).
10. Gey, G.O., Cooper, K.H. and Bottenberg, R.A. Effect of Ascorbic Acid on Endurance Performance and Athletic Injury. *J. Am. Med. A.* 211, 105 (1970).
11. Keren, G. and Epstein, Y. The Effect of High Dosage Vitamin C Intake on Aerobic And Anaerobic Capacity. *J. Sports Med.* 21, 145 (1980).
12. Keith, R.E. and Driskell, J.A. Lung Function and Treadmill Performance of Smoking and Nonsmoking Males Receiving Ascorbic Acid Supplements. *Am. J. Clin. Nutr.* 36, 840 (1982).
13. Simonsson, B. Effect of Cigarette Smoking on the Forced Expiratory Flow Rate. *Am. Rev. Res. Dis.* 85, 534 (1962).
14. Guyatt, A.R., Berry, G., Alpers, J.H., Bramley, A.C. and Fletcher, C.M. Relationship of Airway Conductance and Its Immediate Change on Smoking to Smoking Habits and Symptoms of Chronic Bronchitis. *Am. Rev. Res. Dis.* 101, 44 (1970).
15. Kerrigan, R., Jain, A.C. and Doyle, J.T. The Circulatory Response to Cigarette Smoking at Rest and after Exercise. *Am. J. Med. Sci.* 225, 113 (1968).
16. Allison, R.D. and Roth, G.M. Central and Peripheral Vascular Effects during Cigarette Smoking. *Arch. Environ. Health* 19, 189 (1969).
17. Chevalier, R.B., Bowers, J.A., Bondurant, S. and Ross, J.C. Circu-

NUTRITION REPORTS INTERNATIONAL

- latory and Ventilatory Effects of Exercise in Smokers and Nonsmokers. *J. Appl. Physiol.* 18, 357 (1963).
18. Blackburn, H, Brozek, J., Taylor, H.L. and Keys, A. Comparison of Cardiovascular and Related Characteristics in Habitual Smokers and Nonsmokers. *Ann. N. Y. Acad. Sci.* 90, 277 (1960).
19. Krumholz, R.A., Chevalier, R.B. and Ross, J.C. Cardiopulmonary Function in Young Smokers. *Ann. Inter. Med.* 69, 603 (1964).
20. Keith, R.E. and Driskell, J.A. Effects of Chronic Cigarette Smoking on Vitamin C Status, Lung Function, and Resting and Exercise Cardiovascular Metabolism in Humans. *Nutr. Rep. Int.* 21, 907 (1980).
21. Brook, M. and Grimshaw, J.J. Vitamin C Concentration of Plasma and Leukocytes as Related to Smoking Habit, Age, and Sex of Humans. *Am. J. Clin. Nutr.* 21, 1254 (1968).
22. Pelletier, O. Vitamin C Status of Cigarette Smokers and Nonsmokers. *Am. J. Clin. Nutr.* 23, 520 (1968).
23. Pelletier, O. Vitamin C and Cigarette Smokers. *Ann. N. Y. Acad. Sci.* 258, 156 (1975).
24. Hellman, L. and Burns, J.J. Metabolism of L-Ascorbic Acid-1-C¹⁴ in Man. *J. Biol. Chem.* 230, 923 (1958).
25. Christakis, G., ed. Nutritional Assessment in Health Programs. Public Health A., Washington, DC, 1973.
26. Church, C.F. and Church, H.N. Food Values of Portions Commonly Used, Bowes & Church. J.B. Lippincott Co., Philadelphia, PA, 1975.
27. Sokal, R.R. and Rohlf, F.J. Biometry. W.H. Freeman & Co., San Francisco, 1969.
28. Marcq, M. and Minette, A. Lung Function Changes in Smokers with Normal Conventional Spirometry. *Am. Rev. Res. Dis.* 114, 723 (1976).
29. Friedman, G.J., Sherry, S. and Ralli, E.P. The Mechanism of the Excretion of Vitamin C by the Human Kidney at Low and Normal Plasma Levels of Ascorbic Acid. *J. Clin. Invest.* 19, 117 (1941).
30. Singur, O.I. Pokazатели Utomlenija Učavvihsja pri Različnom Obespečenii organizma Askorbinovoj kislotoj. *Geigiena Sanit.* 7, 117 (1966).
31. Harper, A.A., Mackay, I.F.S., Raper, H.S. and Camm, G.L. Vitamins and Physical Fitness. *Br. Med. J.* 1, 243 (1943).
32. Spoich, F., Kobza, R. and Mazur, B. Influence of Vitamin C upon Certain Functional Changes and the Coefficient of Mechanical Efficiency in Human, during Physical Effort. *Acta Physiol. Polon.* 17, 204 (1966).
33. Ramp, W.K. and Thornton, P.A. The Effect of Ascorbic Acid on the Glycolytic and Respiratory Metabolism of Embryonic Chick Tibias. *Calc. Tissue Res.* 2, 77 (1968).
34. Nitzescu, I.I., Marinescu, G., Gardev, M., Ozun, R., Popa, A. and Gavrilă, I.M. Laktazidämie und Askorbinsäure in der Herzinsuffizienz. *Ztschr. Ges. Inn. Med.* 14, 458 (1959).

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RESPONSE OF RATS TO LYSINE DEFICIENCY AT DIFFERENT AGES.

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ABSTRACT

The effect of feeding a lysine deficient diet to rats of different ages was studied. 3, 13, 26 and 52 week old rats were fed a wheat diet which is deficient in lysine, or the same supplemented with lysine for 8 weeks. The younger animals were more adversely affected by lysine deficiency than the older animals, as judged by growth, blood hemoglobin, serum protein and liver protein. The older animals seemed to adapt to lysine deficiency by reduced turnover of protein or increased reutilization of amino acids as judged by labelling studies. In the second part of the experiment, 3 week old male rats were fed wheat or wheat + lysine diets for 20 weeks in order to study the effects of prolonged feeding of these diets. Thereafter, half the wheat fed animals were switched to a lysine supplemented diet and half the wheat + lysine fed animals were switched to a wheat diet for a period of 12 weeks. Growth was permanently impaired as a result of prolonged lysine deprivation. Adaptability to low lysine, as judged by labelling studies, was lost when the animals were fed lysine deficient diets throughout the period of rapid growth.

INTRODUCTION

It is well known that lysine requirement changes with age, being greater for the young animal than for the adult animal [1]. Lysine requirement may also be affected by the development of adaptive mechanisms which conserve amino acids when they are in short supply [2-3]. Absence of specific amino-acid deficiencies on diets deficient in methionine, cystine, tryptophan and histidine have been reported in adult Indian groups habitually consuming these diets [4]. This could be consequent to development of adaptive mechanisms. The degree of adaptation varies for different amino acids [3]. The adaptive response represents modifications in the catabolic rate of lysine [5], its conservation in the liver as a result of high reutilization [6], modification of the urea cycle enzymes in the liver [7] or reduction in the synthesis of all liver proteins [8].

In addition to these, a general adaptation to low protein diets, mediated through altered rates of tissue protein anabolism and/or catabolism has been reported [9-12].

Since lysine requirements change with age, it could be expected that the adaptation mechanisms that develop in response to a deficiency also vary with age. The first part of the present studies were designed to investigate the response of rats to lysine deficiency, induced at different ages, by feeding them wheat based diets. Estimates of growth, blood hemoglobin, serum protein, liver protein and rate of loss of incorporated labelled amino acid from serum protein were obtained.

The importance of previous dietary history in determining protein requirements has also been reported [12, 13]. Hence in the second part of the study, animals were fed the specified diets from weaning onwards for a prolonged period and comparisons of the various parameters were made with age matched animals who had been fed the diets for short periods in the first part of the study.

METHODS

Albino rats of the Charles-Foster strain, bred in the departmental stock colony were used for these studies. The animals were housed individually in small galvanised iron cages (8"X6"X8").

Experiment 1: Groups of male rats aged 3, 13, 26 or 52 weeks were fed either a wheat diet or the same supplemented with lysine for 8 weeks. The ages were chosen to correspond to periods of rapid growth, puberty, early adulthood with slowed down growth and late adulthood after cessation of significant growth. At the end of the experiment tail blood samples were analysed for blood hemoglobin, serum protein and incorporation of U- 14 C-DL leucine into serum protein. The animals were killed at the end of the treatment for estimation of liver protein.

Experiment 2: Weanling male rats were fed either a wheat diet or the same supplemented with lysine for a period of 20 weeks. At the end of this period, data on blood hemoglobin, serum protein and incorporation of U- 14 C-DL leucine into serum protein were obtained. Half of the animals were then switched over to the diet of the other group i.e. from wheat to wheat + lysine and vice versa, and the experiment terminated after a further period of 12 weeks.

The composition of the diets used is given in Table 1.

Blood hemoglobin and serum protein were estimated from tail blood by the methods described by Varley [14]. For studying the incorporation of a labelled amino acid into serum protein the method of Rao and Radhakrishnan [9] was used after suitable modifications. Counting was done in a liquid scintillation counter by the method of Hall and

Table 1: Composition of the wheat and wheat + lysine diets.

Ingredient g per 100 g diet	Wheat diet	Wheat+lysine diet
Wheat (<i>Triticum sativum</i>)	87	87
Vitamin mixture ¹	2	2
Salt mixture ²	4	4
Groundnut oil	7	7
L-lysine	--	0.2

¹ Vitamin mixture contained per 20g: thiamine, 1.5mg; ribo-flavin, 2.5mg; pyridoxine hydrochloride, 1.0mg; niacin, 15mg; calcium d-pantothenate, 10mg; choline chloride, 750mg; inositol, 200mg; cyanocobalamine, 0.005mg; biotin, 0.001mg. Powdered sugar was added to make a total weight of 20g.

² Salt mixture contained g per 1000g: calcium citrate, 308.2; calcium dihydrogen phosphate, 112.8; dipotassium hydrogen phosphate, 218.7; potassium chloride, 124.7; sodium chloride, 77.0; calcium carbonate, 68.5; magnesium sulfate (anhydrous), 38.3; magnesium carbonate, 35.1; salt mixture 'A', 16.7.

Salt mixture 'A' contained g per 100g: Ferrous ammonium citrate, 91.41; copper sulfate, 5.98; sodium flouride, 0.76; manganese sulfate, 1.07; potassium aluminium sulfate, 0.54; potassium iodide, 0.24.

Cocking [15]. The procedure followed is described in an earlier paper [12]. Specific activity was calculated as counts per minute (cpm) per g serum protein. Liver protein was estimated by the method of Lowry et al [16].

RESULTS AND DISCUSSION

Growth curves of the various groups of experiment 1 (fig 1) confirm that the younger animals are more adversely affected by lysine deficiency. The effect is maximal in the 3 week old lysine deficient group, followed to a lesser extent by the 13 week old wheat fed group. Lysine supplementation to wheat had no demonstrable effect on growth of older animals, viz. the 26 and 52 week old animals. Prolonged feeding of the lysine deficient diet in experiment 2 (fig 2) resulted in marked growth retardation, probably irreversible. Growth of both the groups slowed down at about the same time, indicating absence of adaptation with prolonged treatment. Further, switching of animals from wheat to wheat + lysine or vice versa, 20 weeks after treatment on the initial diets did not result in significant differences in growth rates.

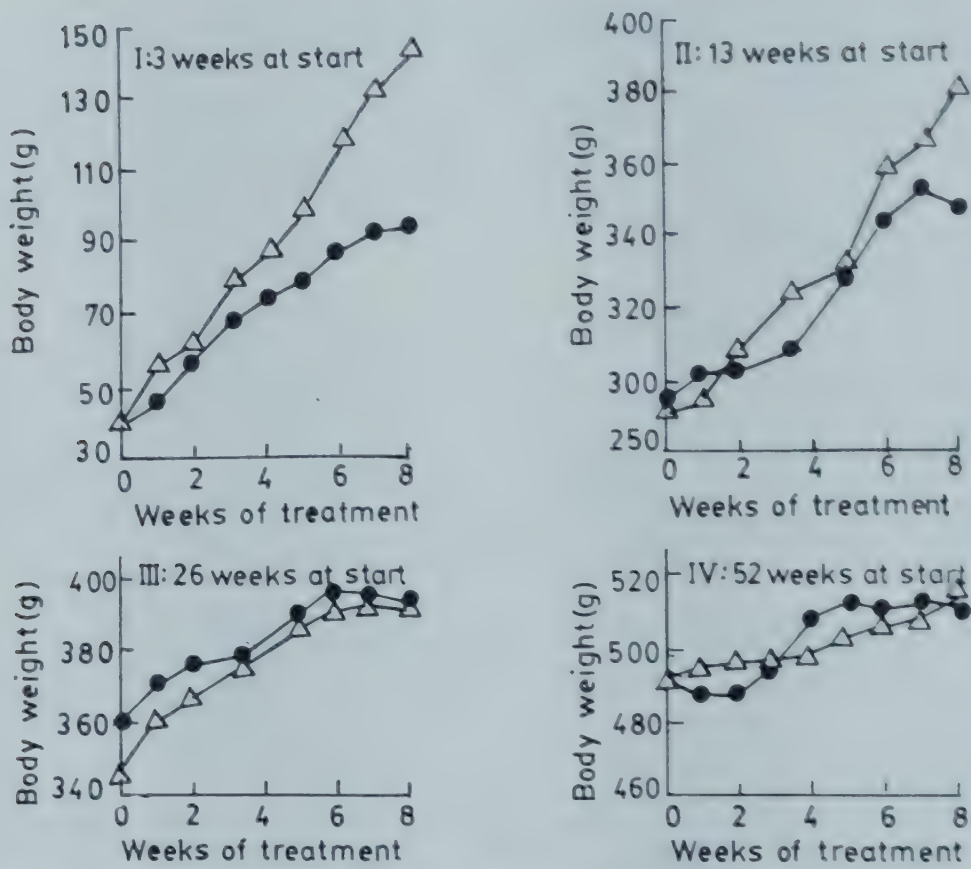


Fig.1: Growth of rats fed wheat (—●—) or wheat + lysine (—△—) based diets at different ages.

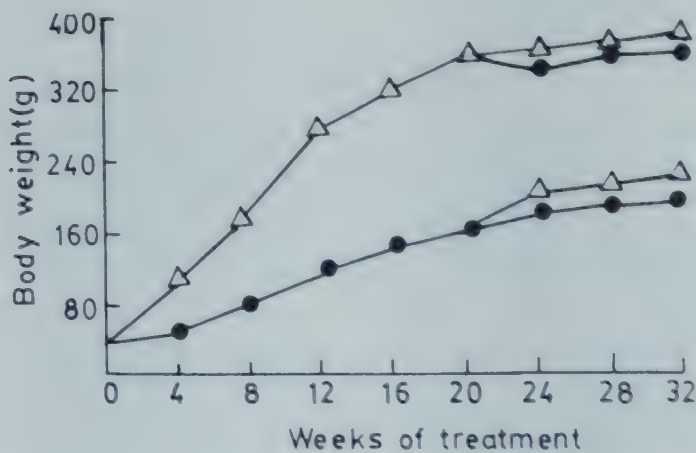


Fig.2: Growth of rats fed wheat (—●—) or wheat + lysine (—△—) based diets from weaning onward for the periods specified.

Blood hemoglobin, serum protein and liver protein were estimated at the end of the treatment. Results are presented in Table 2.

Table 2. Blood hemoglobin, serum protein, and liver protein of rats fed a wheat diet with or without addition of lysine.

Age (weeks)		Diet	Blood Hemoglobin g/100 ml	Serum protein g/100 ml	Liver protein g/100 g
Initial	Final				

mean + s.e. ¹					
3	11	W ²	10.0+0.5 ^a	5.90+0.07 ^b	16.1+0.6 ^c
		W+L	12.7+0.4 ^a	6.20+0.10 ^b	25.1+0.4 ^c
13	21	W	10.3+0.3 ^d	6.25+0.05 ^e	18.6+0.8 ^f
		W+L	11.9+0.4 ^d	6.51+0.05 ^e	24.3+0.6 ^f
26	34	W	11.6+0.6	6.29+0.09	22.8+0.4
		W+L	11.1+0.5	6.46+0.17	23.5+0.9
52	60	W	12.4+0.5	6.33+0.15	20.3+0.6 ^g
		W+L	11.9+0.3	6.60+0.15	23.6+1.0 ^g
3	23	W	11.2+0.2 ^h	6.28+0.12 ⁱ	
		W+L	12.6+0.3 ^h	6.64+0.07 ⁱ	

¹ Based on 6-8 observations. Values marked with the same letter are significantly different from each other at $p < 0.05$.

² W - wheat based diet; W+L - wheat based diet supplemented with lysine.

Blood hemoglobin, serum protein and liver protein were reduced in the 3 week group as a result of lysine deficiency. A similar effect, but of a smaller magnitude was seen in the 13 week old group. Liver protein concentration decreased as a result of lysine deprivation in all age groups, though the decrease did not reach significance for the 26 week old group. Animals subjected to lysine deprivation for 20 weeks in experiment 2 also showed significantly lower blood hemoglobin and serum protein. The magnitude of deficits in blood hemoglobin and serum protein are maximum when lysine deficiency is induced at an early age (3 weeks) for a relatively short period (8 weeks). The deficits, especially with regard to blood hemoglobin are considerable even when lysine deficiency is induced for a short period (8 weeks) at the age of 13 weeks. The deficits decrease in the case of animals of experiment 2 suggesting some adaptation to the wheat diet with prolonged treatment.

The results presented in Table 3 on the incorporation of U- ^{14}C -DL-leucine into serum protein show that at the end of the treatment, lysine supplementation to wheat did not affect the rate of loss of counts from serum protein in the 3 week old animals. However, in the remaining groups, the loss of labelled leucine from serum protein seemed to be slower in all the wheat fed animals as is evidenced from the higher counts found in the serum protein of these groups, as compared to their wheat + lysine counterparts. Significant differences persisted only for a few hours, but the trend seems consistent. In experiment 2, animals fed wheat or wheat + lysine diets for prolonged periods (3-23 weeks) showed no differences with regard to the rate of loss of labelled leucine from serum protein.

Table 3: Incorporation of ^{14}C -DL-leucine into serum protein of rats fed wheat or wheat + lysine diets at different ages.

Age at start +period of treatment (wks)	Diet	cpm per g serum protein at hours after injection						
		12	18	24	48	60	86	105
mean \pm s.e. ¹								
3+8	W ²	2351 \pm 236	1703 \pm 72	1353 \pm 232	1115 \pm 78	842 \pm 63	467 \pm 31	371 \pm 97
	W+L	2381 \pm 89	1511 \pm 52	1272 \pm 58	1030 \pm 61	648 \pm 49	439 \pm 37	338 \pm 28
13+8	W	2401 \pm 67	2021 \pm 85	1891 \pm 69	1392 \pm 70	1187 \pm 78	811 \pm 58	529 \pm 37
	W+L	2118 \pm 71	1480 \pm 100**	1364 \pm 33**	1095 \pm 26*	1010 \pm 29	805 \pm 24	691 \pm 18
26+8	W	2287 \pm 75	1889 \pm 39	1752 \pm 41	1500 \pm 51	1451 \pm 60	878 \pm 45	385 \pm 30
	W+L	2348 \pm 74	1860 \pm 71	1740 \pm 59	1239 \pm 30*	1119 \pm 29*	753 \pm 28*	562 \pm 21
52+8	W	2038 \pm 89	1768 \pm 89	1610 \pm 69	1389 \pm 29	1140 \pm 18	820 \pm 38	480 \pm 41
	W+L	2061 \pm 79	1661 \pm 50	1478 \pm 39	1241 \pm 50*	722 \pm 28**	630 \pm 28*	348 \pm 40*
3+20	W	2102 \pm 123	1719 \pm 95	1337 \pm 67	980 \pm 56			
	W+L	2194 \pm 13	1726 \pm 115	1258 \pm 118	884 \pm 62			

^a Based on 6-8 observations. One and two asterisks respectively indicate significance at $p < 0.05$ and $p < 0.01$.

² W - Wheat based diet.

W+L - Wheat based diet supplemented with lysine.

The overall picture that emerges from the above is that the effects on growth of feeding a low lysine diet are maximum and irreversible when the deficiency is induced at an early age. When the deficient diet is fed in adulthood or later, the effects of lysine deficiency are not visible. However, some covert effects, like the lower liver protein

levels of the low lysine fed 52 week old animals, inspite of normal blood hemoglobin and serum protein levels suggests that some deficiency may indeed be present. Adaptation to this deficiency is suggested by the differences in radioactivity of the wheat and wheat + lysine groups. The pattern observed indicated a slower loss of labelled leucine from serum protein in the wheat fed older animals as compared to their wheat + lysine fed counterparts. The label was injected on the basis of body weight and the counts calculated per g serum protein to help eliminate differences in body size and serum protein levels. It would therefore be reasonable to attribute the differences in radioactivity to differences in synthesis or catabolism of serum protein or reutilization of amino acids.

This ability to adapt seems to be lost if the animal is subjected to lysine deficiency from a young age, as is evidenced by the absence of differences in label between groups of experiment 2. It may be emphasised here that this study does not purport to measure turnover rates of serum protein for reasons discussed in an earlier paper [12]. However, a comparison of the patterns of loss of label obtained in the various groups yields useful results.

The absence of adaptation with regard to growth as a result of prolonged lysine deprivation contrasts with the report of adaptation to low protein diets [17] and with the observation of Osborne and Mendel [18] who found resumption of growth in animals on feeding of complete protein diets after being fed tryptophan deficient maize diets for prolonged periods. However, it must be remembered that different adaptive mechanisms operate to conserve different essential amino acids when they are in short supply [5] so that what may be true for tryptophan may not hold for lysine.

The difference in adaptability of animals differing in age observed in the present study corroborate the fact that age is a critical factor in the utilization of poor quality proteins as is suggested by several studies reviewed by Irwin and Hegsted [19]. This adaptation may be a consequence of reduced turnover of protein [9-12] or increased reutilization of amino acids [20].

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References

1. McLaughlan, J.M. Effects of protein quality and quantity on protein utilization. In 'Newer methods of nutritional biochemistry'. (A.A. Albanese, editor.) Acad. Press, N.Y, (1963) volume 1, p 33.

NUTRITION REPORTS INTERNATIONAL

2. Yamashita, K and Ashida, K. Lysine metabolism in rats fed lysine free diet. *J. Nutr.* 99: 267, (1969).
3. Said, A.K. and Hegsted, D.M. Response of adult rats to low dietary levels of essential amino acids. *J. Nutr.* 100: 1363, (1970).
4. Chitre, R.G., Dixit, M., Apte, V. and Vilekar, V. The concept of essential amino acids in human nutrition. A need of reassessment. *Indian J. Nutr. Dietet.*, 13(4): 101, (1976).
5. Chu, S.W. and Hegsted, D.M. Adaptive response of lysine and threonine degrading enzymes in adult rats. *J. Nutr.*, 106: 1089, (1976).
6. Garlick, P.J., Millward, D.J., James, W.P., and Waterlow, J.C. The effect of protein deprivation and starvation on the rate of protein synthesis in tissue of the rat. *Biochem. Biophys. Acta*, 414: 71, (1975).
7. Das, T.K. and Waterlow, J.C. The rate of adaptation of urea cycle enzymes amino transferase and glutamate dehydrogenase to changes in dietary protein intake. *Br. J. Nutr.*, 32(2): 353, (1974).
8. Canfield, L.M. and Chytil, F. Effect of low lysine diet on rat protein metabolism. *J. Nutr.*, 108(8): 1343, (1978).
9. Rao, B.S.N. and Radhakrishnan, M.R. Incorporation of glycine-1-¹⁴C into tissue of young and adult rats after protein depletion and repletion. *Indian J. Biochem.*, 3(1): 40, (1966).
10. Nettleton, J.A. and Hegsted, D.M. The effects of protein and calcium restriction on tissue nitrogen content and protein catabolism. *Nutr. Metab.*, 17: 166, (1974).
11. Peng, Y., Meliza, L.L., Vavich, M.G. and Kemmerer, A.R. Changes in food intake and nitrogen metabolism of rats while adapting to a low or high protein diet. *J. Nutr.*, 104: 1008, (1974).
12. Mittal, P.C. Response of rats to variations in dietary protein content. *Nutr. Rep. Int.*, 31(3), (1985).
13. Hegsted, D.M. Assessment of nitrogen requirements. *Am. J. Clin. Nutr.*, 31: 1669, (1978).
14. Varley, H. *Practical Clinical Biochemistry*. ELBS and Wm Heinemann Medical Book Ltd. (1969).
15. Hall, T.C. and Cocking, E.C. High efficiency of liquid

NUTRITION REPORTS INTERNATIONAL

scintillation counting of ^{14}C -labelled material in aqueous solutions and determination of specific activity of labelled protein. *Biochem. J.*, 96: 626, (1965).

16. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with folin-phenol reagent. *J. Biol. Chem.*, 193: 265, (1951).
17. Turner, M.R. Protein deficiency, reproduction and hormonal factors in growth. *Nutr. Rep. Int.* 7(5): 289, (1973).
18. Osborne and Mendel. *J. Biol. Chem.*, 23, 439 (1915) cf. 'Nutrition' Eds. Beaton, G.H. and McHenry, E.W. Vol III. Acad. Press, N.Y. (1966).
19. Irwin, M.I. and Hegsted, D.M. A conceptus of research on amino acid requirements of man. *J. Nutr.*, 101: 539, (1971).
20. Dallman, P.R. and Manies, E.C. Protein deficiency. Turnover of protein and reutilization of amino acids in cell fractions of rat liver. *J. Nutr.*, 103: 257, (1973).

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EFFECT OF COPPER, COBALT AND ZINC SUPPLEMENTATION ON LIVELINE GAIN OF NELLORE HEIFERS IN THE PERUVIAN TROPICS¹

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ABSTRACT

Aqueous solutions of copper, cobalt and zinc were given to 32 Nellore heifers grazing tropical grasses, Hyparrhenia rufa and Paspalum conjugatum, during seven months at the Tropical Research Station in Pucallpa, Perú. A 2x2x2 factorial experiment was used with the following treatments: 1) control; 2) copper; 3) cobalt; 4) zinc; 5) copper and cobalt; 6) copper and zinc; and 7) copper, cobalt and zinc. Daily doses were as follows: copper, 45 mg; cobalt, 7.2 mg; and zinc, 225 mg. All heifers received 4.5 g of phosphorus per day to correct this deficiency found in previous research. Liveweight gains were 200, 213, 151, 188, 149, 181, 117 and 166 g/day for copper, cobalt, zinc, copper-cobalt, copper-zinc, cobalt-zinc, copper-cobalt-zinc and control groups, respectively. Statistical differences ($P < 0.1$) were found, with economic benefits resulting from providing copper and cobalt as supplements. Zinc supplementation did not improve liveweight gains.

INTRODUCTION

In tropical Latin America, mineral deficiencies and imbalances are severely inhibiting cattle production (1, 2). In Perú, tropical grasses were found to be deficient in phosphorus, and supplementation with this nutrient increased liveweight gains of Nellore heifers (3). Copper and cobalt have also been reported deficient in native grasses from the Upper Amazon basin of Perú (4). Likewise, authors of the present paper have observed lameness, nonspecific anemia, broken bones and skin alterations in Zebu cattle from the same region. The present experiment was designed to determine the effects of copper, cobalt and zinc supplementation on liveweight gains of Nellore heifers in the Peruvian tropics.

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METHODS

A supplementation study was carried out at the principal research station of I.V.I.T.A. (Instituto Veterinario de Investigaciones Tropicales y de Altura) in Pucallpa, Perú. This station is located at longitude 75°05'W and latitude 8°02'S at about 280 m above sea level. Average temperature is 26°C, with annual rainfall of 1700 mm distributed in two periods, high rainfall season (November - May) and reduced rainfall season (May - November). The experiment lasted seven months, from May to November, to cover the reduced rainfall season when pasture generally decreases in yield and quality.

Thirty-two Nellore heifers, 20 ± 2 months old and averaging 178 ± 15 kg, were used with animals grazing Hyparrhenia rufa and Paspalum conjugatum without access to minerals, including common salt. A factorial experiment of $2 \times 2 \times 2$ was used with four animals per treatment. Treatments were as follows: 1) control; 2) copper; 3) cobalt; 4) zinc; 5) copper-cobalt; 6) copper-zinc; 7) cobalt-zinc; and 8) copper-cobalt-zinc. Mineral sources were reagent grade and were supplied orally once a week as an aqueous solution using plastic syringes in order to provide the requirements for copper, cobalt and zinc in cattle as suggested by Underwood (5). Supplied levels were as follows: 45 mg Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 1.2 mg Co ($\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) and 225 mg Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) per animal daily. In addition, 4.5 g of phosphorus ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) were administered to all heifers to correct reported phosphorus deficiency (4). Heifers were weighed monthly after a 15-hour fast. Covariance analysis and analysis of variance were carried out to compare treatments for the final weight gains only (6).

RESULTS AND DISCUSSION

Monthly and total liveweight gains for the eight treatment combinations are presented in Table I, and Figure 1 graphically illustrates the main treatment effects. The overall mean gain for the seven-month period was 35.9 kg. No significant ($P > 0.05$) differences were found among treatments for the final weight gains. Although not significant at the 5 percent significance level, this level of significance was approached by heifers on treatments of cobalt, copper and cobalt-copper, with weight gains higher ($P < 0.10$) than control animals.

Cumulative liveweight gains were not corrected because pre-experimental liveweights were found not to be significantly correlated with final weight and cumulative weight gain regression coefficient was not statistically ($P > 0.05$) significant. Lack of a difference at the 5 percent significance level for cobalt and copper treatments is attributed to the small number of experimental animals. For grazing livestock in Latin America, McDowell et al. (7) reports that, with the exception of phosphorus inadequacy, deficiencies of copper and cobalt are the two trace mineral inadequacies most likely limiting to grazing livestock. On the other hand, heifers fed zinc did not exhibit increased weight gains compared to the control treatment group. It is possible to speculate that zinc, when combined with copper or with both copper and cobalt, lowered gains, attributed to zinc reducing absorp-

TABLE I. LIVEWEIGHT GAINS OF NELLORE HEIFERS SUPPLEMENTED WITH COPPER, COBALT AND ZINC^a

Treatment	Accumulative weight gain by month (kg)						Daily mean liveweight gain (kg)
	1	2	3	4	5	6	7
Control	11.8	17.8	17.5	23.8	24.8	33.5	34.8
Copper	14.5	23.0	27.0	30.5	33.5	38.5	42.0
Cobalt	15.5	20.3	28.1	28.1	32.6	37.1	44.7
Zinc	14.8	14.8	15.3	16.1	22.1	26.4	31.9
Copper + cobalt	21.0	27.0	32.2	33.7	33.2	27.3	39.5
Copper + zinc	11.3	17.8	22.5	22.0	25.8	27.3	31.3
Cobalt + zinc	9.5	17.0	23.5	30.7	30.0	36.2	38.0
Copper + cobalt + zinc	12.2	15.0	18.2	23.0	24.0	29.2	24.5

^aNo significant difference among treatments ($P > 0.05$).

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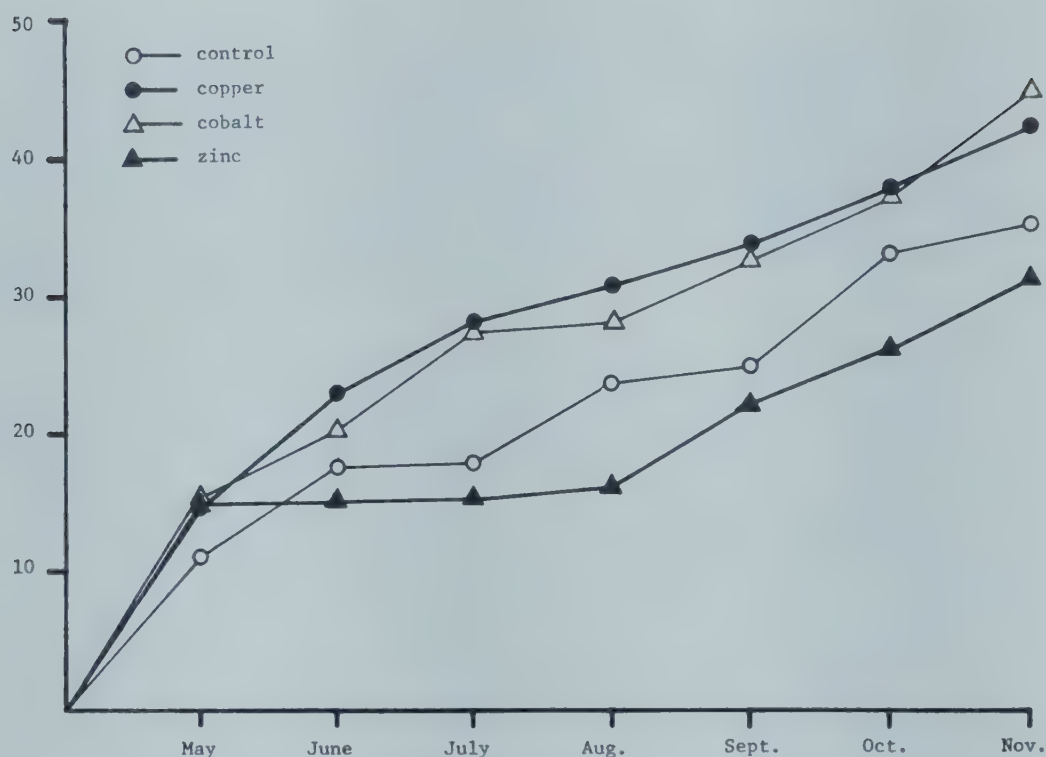


Figure 1.

Mineral treatment effects on liveweight gains of supplemented heifers.

tion of copper (8) due to both minerals having the same tetrahedral electronic configuration competing for intestinal absorption, and the enzymatic activity in mineral metabolism.

From Perú the tropical grasses Hyparrhenia rufa and Paspalum conjugatum were reported as deficient in copper (4) in relation to the requirements for grazing cattle (5). Copper and cobalt used as constituents of mineral supplements have increased growth rates in other tropical areas (9, 10, 11). Similarly, the results from the experiment reported herein indicated increases in liveweight gains for cattle grazing tropical grasses.

CONCLUSIONS

This study showed increased ($P < 0.10$) growth rates in Nellore heifers fed copper and/or cobalt. The economic benefits obtained from the liveweight gains suggest that copper and cobalt supplementation be recommended in Pucallpa, Perú. No benefit, however, was found from zinc supplementation.

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REFERENCES

1. McDowell, L.R. Mineral deficiencies and toxicities and their effects on beef production in developing countries. *In* Beef Cattle Production in Developing Countries (A.J. Smith, editor). University of Edinburgh, Centre for Tropical Veterinary Medicine, Scotland, pp. 216-241 (1976).
2. McDowell, L.R. and Conrad, J.H. Simposio Latinoamericano Sobre Investigaciones en Nutrición Mineral de los Rumiantes en Pastoreo. University of Florida, Gainesville (1978).
3. Echevarria, M., Valdivia, R., Santherazegaram, K., Campos, L. and Arbeiza, T. Efecto de la suplementación fósforo sobre los niveles séricos y crecimiento de vaquillas. *Revista de Investigaciones Agropecuarias*, 82-85 (1978).
4. Beeson, K.C. and Gomez, G. Concentration of nutrients in pastures in the Central Huallaga and Río Ucayali Valleys of the upper Amazon basin of Perú. *Proceedings of XI International Grasslands Congress*, 89-92 (1980).
5. Underwood, E.J. The Mineral Nutrition of Livestock. Commonwealth Agricultural Bureaux, London (1981).
6. Steel, R.G.D. and Torrie, J.H. Principles and Procedures of Statistics (2nd edition). McGraw-Hill Book Company, Inc., New York (1980).
7. McDowell, L.R., Conrad, J.H. and Ellis, G.L. Mineral deficiencies and imbalances and their diagnosis. *In* Herbivore Nutrition in Subtropics and Tropics (F.M.C. Gilchrist and R.I. Mackie, editors), pp. 67-88. The Science Press, Pretoria, Republic of South Africa (1984).
8. Hill, C. and Matrone, G. A study of copper and zinc interrelationships. *Proceedings of XIII World Poultry Congress*, 219 (1962).
9. Donaldson, L.E., Harvey, J.M., Beattie, W.A., Alexander, G.I. and Burns, J.A. Effects of copper and cobalt supplementation on growth rate and fertility of shorthorn heifers in Northern coastal Queensland. *J. Agric. Sci.* 21, 167-178 (1964).
10. Howard, D. The effects of cobalt and copper treatment in the weight gains and blood constituents of cattle in Kenya. *Veterinary Record* 87, 771-774 (1970).

NUTRITION REPORTS INTERNATIONAL

11. Winter, W.H., Siebert, B.D. and Kuchel, R.E. Cobalt deficiency of cattle grazing improved pasture in Northern Cape York Peninsula. *Australian J. Exp. Agric. Anim. Husb.* 17, 10-17 (1977).

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SUPPRESSION OF CONCAVALIN-A MEDIATED BLASTOGENESIS OF SPLEEN AND LYMPHNODE LYMPHOCYTES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS AND MICE

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ABSTRACT

Lymphocytes isolated from spleens and lymphnodes of streptozotocin-induced diabetic mice and rats were significantly less responsive to Concanavalin A stimulation than those isolated from control animals. The spleen weights of the diabetic rats were reduced. When insulin treatment were given to diabetic mice, the effect of diabetes in suppressing lymphocytic responses could be partially prevented.

INTRODUCTION

Increased incidence of infection are common in poorly controlled diabetics (1). The diabetic state is believed to decrease the cell-mediated immune response in patients and experimental animals. For examples, diabetic patients were reported to have a diminished peripheral blood lymphocyte response to phytohemagglutinin (2) and antigen of staphylococcus (3). Diabetic mice induced with streptozotocin or pancreatectomy showed reduction of inflammatory response, granuloma formation and delayed hypersensitivity reaction to tuberculin (4). It has also been shown that the absolute peripheral leukocyte and spleen cell numbers of streptozotocin-induced diabetic rats were significantly decreased (5). Although the nature of the suppression of immunity in diabetics is still obscure, several studies investigating streptozotocin-induced diabetic mice have suggested that the immune suppression may be resulted from the decreases in weights (6) and numbers of nucleated cells (7) (8) (9) in lymphoid organs. The purpose of this study is to determine whether streptozotocin-induced diabetes might have any effect on the lymphocytes of the spleen and the lymphnodes of rats and mice by using in vitro Concanavalin A (Con A) stimulation assay.

METHODS

Male Sprague-Dawley rats weighing about 300 g and female inbred Balb/c mice weighing about 25 g were used. A single dose of streptozotocin (SIMGA) were administered IP to rats (100 mg/kg body weight) and mice (200 mg/kg body weight) to induce diabetes. The respective controls received saline only. All animals were fed ad

libitum commercial pellets and housed in plastic cages in a conventional environment at $22^{\circ} \pm 1^{\circ}\text{C}$ and light 12 hours.

Four days after streptozotocin injection, rats and mice were sacrificed and their spleens were removed and weighed. The spleens of rats were individually suspended in RPMI 1640 culture medium (KC BIOLOGICAL) supplemented with 25 mM HEPES buffer (pH 7.4), 200 mM L-glutamine, penicillin-streptomycin (100 units/ml), mycostatin (0.2 ml/100 ml), gentamycin (20 $\mu\text{g/ml}$) and 10% (v/v) heat-inactivated fetal calf serum (GIBCO). The spleens of inbred mice were pooled together and suspended in the above culture medium. Tissues from rats or mice were gently teased and filtered through gauze. The spleen cells were washed twice by centrifugation. The viability of the spleen cell preparations as determined by trypan blue exclusion ranged about 90% in all cases. Lymphocyte suspensions were also prepared from the axillary and inguinal lymphnodes of animals as described before (10). Cell viability also ranged about 90%.

0.18 ml of lymphocyte suspensions (5×10^6 cells/ml) from spleens or lymphnodes were mixed respectively with 0.2 ml Con A (E.Y. LAB.) solution in culture medium in the wells of microplate. In pilot experiments the optimal concentration of Con A for stimulation of lymphocytes of spleens and lymphnodes of rats and mice were determined and these concentrations were used in later experiments. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 48 hours. 0.02 ml ^3H -thymidine (25 $\mu\text{Ci/ml}$ in medium) were then added to each well. Cells were harvested 6 hr. later through GF/C filter papers (WHATMAN) by cell harvester (FLOW LAB.). The radioactivity retained on the filters was determined by liquid scintillation counter (BECKMAN). All incubations were performed in triplicate. The data were reported as the net stimulation (cpm) of the incorporation of ^3H -thymidine in the presence and absence of Con A.

For the insulin treatment of diabetic mice, mice after given injection of streptozotocin received daily SC injections of 2 I.U. insulin (SIMGA) in saline for 4 days. The effect of insulin was monitored by serum glucose of the animals. Serum glucose was determined by glucose oxidase method (SIMGA).

Statistical analysis: student-t test. Significant difference: *, $P < 0.001$.

RESULTS

As indicated in table I, streptozotocin-induced diabetes significantly reduced the spleen weights of rats.

Figure 1 shows the magnitude of response of lymphocytes isolated from spleens and lymphnodes of normal and diabetic rats to Con A. The concentration of Con A used in spleen lymphocyte stimulation was 18 $\mu\text{g/ml}$ and that in lymphnode lymphocyte was 9 $\mu\text{g/ml}$. Diabetes significantly suppressed lymphocytic response in rat and this effect was more pronounced in lymphocytes isolated from the spleen.

Table I. The spleen weights of normal and streptozotocin-induced diabetic rats

Group	Spleen weights (g/Kg body weight)
Control	2.14 ± 0.42
Diabetic	0.85 ± 0.32*

Note: rats were induced diabetes by IP injection of 100 mg/kg body weight streptozotocin in saline. Only saline were injected to control. Four days after administration, both groups were sacrificed and the spleens were weighed. Mean of 5 rats ± SEM.

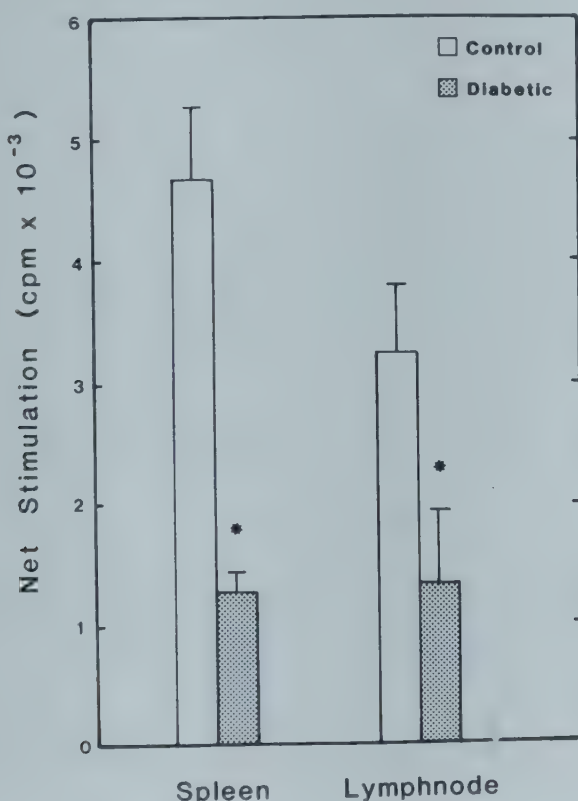


Figure 1. Con A stimulated blastogenesis of control and streptozotocin-induced diabetic rats. Lymphocytes after isolating from spleens and lymphnodes of rats were cultured and stimulated with Con A as described in text. Mean of 5 rats ± SEM.

Streptozotocin can kill the pancreatic β -cells (11) and render the mice diabetic. If the suppression of lymphocytic response to Con A is a direct consequence of diabetes, treatment of diabetes with insulin may neutralize this effect. The results of experiments designed to

test this possibility is shown in table II.

Table II. The effect of insulin on the lymphocytic response to Con A of spleen and lymphnode of diabetic inbred mice

Treatment	Serum glucose (mg/100 ml)	³ H-thymidine incorporation (cpm x 10 ⁻³)	
		spleen lymphocytes	lymphnode lymphocytes
Control	140.3 ± 6.5	6.5 ± 2.8	34.0 ± 1.1
Diabetes	350.5 ± 10.5	0.2 ± 0.05	0.5 ± 0.05
Insulin-treated diabetes	176.4 ± 21.3	5.4 ± 1.4	19.0 ± 6.1

Note: lymphocytes were isolated from 5 inbred Balb/c mice. Lymphocytes obtaining from the same group were pooled and stimulated with Con A as described in text. Mean of 3 determinations ± SEM.

Diabetic mice were treated with insulin by administering SC 2 I.U. insulin per day for 4 days. The lymphocytic response to Con A of control, diabetic and insulin treated diabetic mice were determined. The concentration of Con A used in spleen and lymphnode lymphocytes stimulation were 30 µg/ml. As indicated in table II, insulin treatment suppressed the blood glucose concentration of the diabetic mice nearly to normal. Concerning Con A blastogenesis, diabetes significantly suppressed the response of lymphocytes isolated from both spleens and lymphnodes. However, these suppressions in diabetes could be partially recovered by the administration of insulin. As indicated in table II, the ³H-thymidine incorporations of lymphnode and spleen lymphocytes of insulin-treated diabetic group resumed to about 60%-80% of that of control.

DISCUSSION

The effect of diabetes on the immune system is by and large unknown. By using streptozotocin-induced diabetic mice as an animal model, Ishibashi *et al.* (6) postulated that the suppressive effect of diabetes on immune system may be related to the decrease of spleen weights under diseased condition. We confirmed (table I) that the spleen weights of rats also decreased in streptozotocin-induced diabetes. Spleen is an important lymphoid organ, decrease in spleen weights in diabetes may account for the diminution of lymphocytes in peripheral blood observed in streptozotocin-treated mice (9) and rats (5).

In the present study we have also attempted to investigate the effect of diabetes on the lymphocytic response of spleen and lymphnodes towards Con A stimulation in rats and mice. It is evident that streptozotocin-induced diabetes can suppress the lymphocytes to the stimulation of Con A in both lymphoid organs in rats and mice (figure 1 and table II). The half-life of streptozotocin is about 15 minutes in experimental animals (12)(13). Since there is no evidence demonstrating the existence of streptozotocin binding sites on lymphocytes, it is unlikely that the inhibition of Con A-stimulated blastogenesis is mediated by streptozotocin itself. Since streptozotocin can selectively damage pancreatic β islet cells (11) and render the animal diabetic, it is likely that the proliferation of the lymphocytes may be impaired in diabetic state. To test this hypothesis, we examined the effect of insulin on the blastogenesis of the animals. Daily administration of insulin for 4 days could partially reverse the suppressive effect of streptozotocin-induced diabetes on Con A mediated blastogenesis of lymphocytes of spleens and lymphnodes of mice (table II).

The exact suppressor(s) triggering the diminution of lymphocyte response in diabetic host remains to be identified. Chi *et al.* (5) showed that very-low-density lipoprotein from diabetic rat serum injures lymphocytes and impair the immunity of the streptozotocin-induced diabetic rat. Furthermore, low-density lipoprotein (14) and high-density lipoprotein (15) have recently been found to possess inhibitory activities for mitogen-induced human lymphocyte activation. We (16) also observed that lecithin abrogates blastogenic responses of human lymphocyte cultures stimulated by mitogens and various antigens. It would therefore be interesting to examine the effects of diabetes-induced hyperlipoproteinaemia and hyperlipidaemia on the immunity of the host.

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REFERENCES

1. Bagdade, J.D. Diabetes Mellitus: diagnosis and treatment. (S.S. Fajans and K. Sussman, editors), American Diabetes Association, New York 1971, p.214.
2. Maccuish, A.C., Urbaniak, S.J. and Campbell, C.S. Phytohemagglutinin transformation and circulating lymphocyte subpopulations in insulin-dependent diabetic patients. *Diabetes* 23, 708 (1974).
3. Casey, J.I., Heeter, B.T. and Klyshevich, K.A. Impaired response of lymphocytes of diabetic subjects to antigen of *Staphylococcus aureus*. *J. Infect. Dis.* 136, 495 (1977).

4. Conge, G.-A., Gouache, P. and Lagrange, P.H. Cell-mediated immune response in diabetic mice. *Nutr. Reports Intern.* 25, 443 (1982).
5. Chi, D.S., Berry, D.L., Dillon, K.A. and Arbogast, B.W. Inhibition of in vitro lymphocyte response by streptozotocin-induced diabetic rat serum. *diabetes* 31, 1098 (1982).
6. Ishibashi, I., Kitahara, Y., Harade, Y., Harada, S., Takamoto, M. and Ishibashi, T. Immunologic features of mice with streptozotocin-induced diabetes. Depression of their immune responses to sheep red blood cells. *Diabetes* 29, 516 (1980).
7. Nichols, W.K., Spellman, J.B. and Daynes, R.A. Immune responses of diabetic animals. Comparison of genetically obese and streptozotocin-diabetic mice. *Diabetologia* 14, 343 (1978).
8. Pavelic, K., Slijepcevic, M. and Pavelic, J. Recovery of immune system in diabetic mice after treatment with insulin. *Horm. Metab. Res.* 10, 381 (1978).
9. Nichols, W.K., Spellman, J.B., Vann, L.L. and Daynes, R.A. Immune response of diabetic animals. Direct immunosuppression effects of streptozotocin in mice. *Diabetologia* 16, 51 (1979).
10. Ho, W.K.K. and Leung, A. The effect of morphine addiction on Concanavalin A - mediated blastogenesis. *Pharmacol. Res. Commun.* 11, 413 (1979).
11. Arison, R.N., Ciaccio, E.I., Glitzer, M.S., Cassaro, A.J. and Pruss, M.P. Light and electron microscopy of lesions in rats rendered diabetic with streptozotocin. *Diabetes* 16, 51 (1967).
12. Schein, P.S., Kahn, R., Gorden, P., Wells, S. and DeVita, V.T. Streptozotocin for malignant insulinomas and carcinoid tumour. *Arch. Intern. Med.* 132, 555 (1973).
13. Saiki, O., Negoro, S., Tsuyuguchi, I. and Yamamura, Y. Depressed immunological defense mechanisms in mice with experimentally induced diabetes. *Infec. Immun.* 28, 127 (1980).
14. Curtiss, L.K. and Edgington, T.S. Regulatory serum lipoproteins: regulation of lymphocytes stimulation by a species of low density lipoprotein. *J. Immunol.* 116, 1452 (1976).
15. Hui, D.Y., Harmony, J.A.K., Innerarity, T.L. and Mahley, R.W. Immunoregulatory plasma lipoproteins. Role of apoprotein E and apoprotein B. *J. Biol. Chem.* 24, 775 (1980).
16. Ng, M.H., Ng, W.S., Ho, W.K.K., Fung, K.P. and Lamelin, J.P. Modulation of phytohemagglutinin-mediated lymphocytes stimulation by egg lecithin. *Exptl. Cell Res.* 116, 387 (1978).

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STEEL MAKING SLAG AS A SOURCE OF DIETARY CALCIUM FOR THE LAYING HEN¹

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ABSTRACT

Experiments were conducted for the purpose of evaluating steel making slag as a source of dietary calcium for laying hens. Substitution of slag for limestone in a ration for laying hens resulted in decreased egg production, shell thickness, fertility and hatchability. The slag material appears to be an inferior source of calcium for the laying hen and may also interfere with the utilization of other dietary nutrients.

INTRODUCTION

Since egg shell damage represents a significant economic problem for the poultry industry, many potential sources of dietary calcium have been evaluated. Little information is available concerning the value of by-products of the steel industry as potential sources of dietary calcium. Limestone is utilized in several types of steel making processes resulting in a slag waste product relatively high in calcium content. The purpose of this investigation was to evaluate steel making slag as a source of calcium in laying hen diets since this material is readily available in substantial quantities.

MATERIALS AND METHODS

Two experiments were conducted with laying hens maintained in individual cages. Ten-month-old White Leghorn hens were assigned to the cages based upon prior egg production so that each experimental group of 24 hens started the experiment with the same rate of egg production. Feed and water were supplied ad libitum. The basal diet was a corn-soybean meal ration containing 20 percent protein. Both diets contained 3.88 percent calcium with 3.3 percent calcium being derived from limestone (38 percent calcium) or steel making slag (31 percent calcium). Major constituents of the slag other than calcium were: Fe (16 percent), Si (5.4 percent), and Mg (4.2 percent). Egg production was

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recorded daily. The hens were artificially inseminated with pooled semen weekly. Eggs collected three days per week were evaluated for shell thickness while the eggs from the remaining four days were placed in the incubator to evaluate fertility and hatchability.

RESULTS AND DISCUSSION

The first experiment was terminated after two weeks because the hens fed the diet containing steel-making slag ceased egg production. An evaluation of the situation indicated that feed consumption was low. Since these diets were loose mash-type, the hens were selecting ingredients other than the slag so there was a concentration of this material in the feeder. An analysis of the feed remaining in the feeder suggested an approximate threefold enhancement of dietary mineral content.

Thus, a second experiment was initiated in which the feed was pelleted in order to minimize ingredient selection. The results of this experiment are presented in Table 1. The inclusion of slag in the diet resulted in a significant reduction in egg production and egg shell thickness. Eggs that were incubated showed a significant reduction in fertility and hatchability. Examination of unhatched eggs revealed that there was some embryonic death at a very early age, while most embryos died at 17-18 days of embryonic development. Gross examination of these embryos

Table 1. Influence of Source of Dietary Calcium Upon Production Characteristics of Laying Hens (11 week)

Source of Calcium	Egg Production	Egg Shell Wt/area	Fertility	Hatchability ¹
	%	mg/cm ²	%	%
Limestone	75 (83-68)	78 (81-76)	88 (95-85)	80 (96-70)
Steel making Slag	41* (69-25)	68* (81-59)	55* (87-50)	19* (83-0)

¹Percent of fertile eggs.

²Figures in parenthesis represent the range of values obtained (1st week - 11th week)

*Significantly different from limestone treatment $p < 0.05$.

did not show any particular morphological defects which might give some indication of the cause of embryonic death.

In an attempt to ascertain if there were toxic quantities of minerals in the eggs, the liquid contents of the eggs were lyophilized, lipid-extracted and subjected to mineral analyses. There were not substantial differences between the two groups of eggs in Ca, P, Na, K, Al, Cd, Cr, Cu, Fe, Ni, Pb or Zn content. These results suggest that the impaired embryonic development associated with the slag diet was not due to toxicity of analyzed minerals. Thus, the reason for the lack of embryonic development remains unknown. The most logical explanation would be that the slag was interfering with the absorption of other nutrients and producing a secondary nutrient deficiency. One other observation supports this hypothesis. After the hens had been fed the experimental diets for approximately 2 weeks, there was an obvious reduction in the amount of pigment deposited in the yolks of the hens fed the slag diet. Since the diets were calculated to be identical in pigment content it must be concluded that the slag was interfering with the absorption or metabolism of these substances. Other nutrients could have been similarly affected.

Calcium metabolism was also affected in the hens receiving the steel-making slag as a source of dietary calcium. This is exemplified by the fact that there was a substantial reduction in shell thickness during the course of the experiment. Blood samples taken at the conclusion of the experiment showed a significant reduction (17.0 vs. 23.9 mg%) in serum calcium of the hens fed the slag-containing diets. Bones taken from these hens showed a slight reduction in weight with no reduction in bone ash content. Overall, these results suggest that the slag material used in this study is unacceptable as a source calcium for laying hens. This material differs in composition from the slag studied by Damron *et al.* (1). These investigators found a phosphorus containing basic steel slag to be a poor source of phosphorus and appeared to contain some factor that was toxic to broiler chickens.

REFERENCES

1. Damron, B. L., E. A. Paz and L. R. McDowell. Basic steel slag in the diet of broiler chicks. *Nutr. Rep. Int.* 27:1315-1321 (1983).

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HEAT AFFECTS NUTRITIONAL CHARACTERISTICS OF SOYBEAN MEAL AND EXCRETION OF PROTEINASES IN MINK AND CHICKS

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ABSTRACT

Two soybean products were subjected to moist heat at 110 and 135 °C for 10 and 30 min. to study the comparative effects on amino acid digestibility and trypsin excretion in mink and chicks. Unheated soybean flakes were poorly digested in both species and failed to support normal growth in chicks. Heating to 110 °C greatly improved all nutritional characteristics, whereas heat treatment at 135 °C resulted in loss of lysine, arginine and cystine, and reduced digestibility of all amino acids. When evaluated from the relationship with chick growth, amino acid digestibility was superior to metabolizable energy as indicator of nutritional value. The trypsin activity of mink feces was about 20 times higher than that of chick excreta, increasing with excessive heat treatment. A surplus of fecal trypsin was found in mink fed unheated soybean flakes, while chick excreta contained an excess of proteinase inhibitors. Thus the pancreas of mink and chick appeared to respond differently to dietary proteins and inhibitors.

INTRODUCTION

The occurrence of natural proteinase inhibitors in soybeans has long been recognized. Feeding of raw soybeans causes growth depression and pancreatic hypertrophy in monogastric animals. The soybean proteinase inhibitors are inactivated by proper heat treatment. However, excessive heating may lead to destruction of amino acids or reduced amino acid availability. It is therefore of great importance to achieve a balanced heat treatment, considering the heat needed to destroy the inhibitors as well as the possible adverse effects on nutritionally important amino acids.

Soybean meal is the largest single source of protein in chicken feeding, but plays a minor role in common mink diets. Mink belong to the mammalian order Carnivora, and the digestive capabilities may be different from those of chickens. There is limited information on the comparative response of mink and chicks fed differently heated soybean products. The present experiments were designed to study the effects of different heat treatments on (a) amino acid contents and digestibility when fed to mink and chicks, (b)

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growth performance in chicks and (c) the excretion of proteolytic enzymes in mink and chicks.

MATERIAL AND METHODS

Two soybean products were selected for use as basal raw materials. Desolventized soybean flakes ("white flakes") were obtained from the American Soybean Association, Hamburg, representing an unheated raw material. A commercial solvent extracted soybean meal, already heated to about 110 °C with short-term toasting, was obtained from a Norwegian supplier. Samples of both products were soaked with 20 % water (w/w) and autoclaved. The temperature/time (°C/min.) schedules were for desolventized soybean flakes 110/10, 135/10 and 135/30, and for solvent extracted soybean meal 135/10 and 135/30. After cooking, the samples were cooled and dried at 30 °C and subsequently ground to a powder using a hammer mill with 2 mm sieve.

Digestibility studies with mink were carried out as described earlier, using 4 adult males of the standard breed per treatment (1). In the diets used, the soybean products represented 2/3 of the total protein, the remaining 1/3 was provided as cod fillet. The true digestibilities of N and amino acids were determined using difference calculation and a previously described procedure for calculating the metabolic fecal excretions (1).

Experiments with chickens were carried out using White leghorn male chicks. For determinations of metabolizable energy and amino acid digestibility, 5-8 weeks old chicks were kept in individual cages equipped for measurements of feed intake and collection of excreta. The soybean products, supplemented with vitamins and minerals, were fed ad libitum to 6 birds per treatment for 7 days, excreta being collected the last 4 days. Metabolizable energy values were corrected to zero nitrogen retention. The metabolic excretion of amino acids was estimated from data obtained in previous studies (2, 3). Tryptophan was not analysed. In growth studies, the chicks were fed semipurified diets ad libitum for 28 days, with soybean products as the sole source of protein. Twelve replicate pairs of chicks per treatment were kept in wire pens from day-old. The chicks were kept in a constantly illuminated room at temperatures of 33 °C the first 14 days and 28 °C thereafter.

Proximate analysis of feed ingredients, freeze-dried feces and excreta was carried out as described earlier (1). Amino acid determinations were performed at "Statens Landbrukskemiska Laboratorium", Uppsala, Sweden.

Trypsin activity was analyzed in freeze-dried samples of mink feces and chick excreta (4). Negative values, found

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with chick excreta, indicate that all trypsin is neutralized by inhibitors in the excreta. The surplus of inhibitors has the capacity to inhibit an activity of chicken trypsin as shown by the negative value (4).

The trypsin inhibitor content of the soybean products was analyzed according to the standard analytical method described by Hammerstrand *et al.* (5). Bovine trypsin (No. T-8253, Sigma Chem Co., St. Louis, Missouri) was used as trypsin source.

Statistical analyses

Results were evaluated by analysis of variance and Newman Keul's test. Differences were considered significant when the probability of obtaining them by chance was 5 % or less.

RESULTS

Heat treatment by autoclaving the soybean products at 135 °C caused a yellowish/brownish color. The effect on the contents of trypsin inhibitors is shown in Table 1. The experimental heat treatments reduced the content of trypsin inhibitors to a very low level. The unheated desolventized flakes revealed a high inhibitor activity, whereas the activity of the commercial solvent extracted meal was within the normal range of this type of product.

Table 1. Effect of heat treatment (temp./time) on contents of inhibitors in soybean desolventized flakes and solvent extracted soybean meal (% of contents in unheated desolventized flakes)

Soybean desolventized flakes (°C/min.)				Solvent extracted soybean meal (°C/min.)		
-	110/10	135/10	135/30	-	135/10	135/30
100.0	0.1	0.0	0.2	7.0	0.2	0.3

The proximate composition was not affected by heating. Amino acid composition of the soybean products is shown in Table 2. Heating to 110 °C had no detrimental effect on amino acid contents. By contrast, autoclaving to 135 °C caused a reduction in the contents of lysine, arginine and cystine. The losses were increased by extending the time of heat application from 10 to 30 min. The data of other amino acids showed little or no effects of heat treatment. The experiments revealed great differences between different soybean products as regards acceptability in mink. All mink fed unheated desolventized flakes completely re-

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Table 2. Effect of heat treatment (temp./time) on the amino acid composition of soybean desolventized flakes and solvent extracted soybean meal (g/16 g N)

	Soybean desolventized flakes (°C/min.)				Solvent extracted soybean meal (°C/min.)		
	-	110/10	135/10	135/30	-	135/10	135/30
Lys	6.5	6.3	5.8	5.2	6.6	5.4	5.0
His	2.7	2.6	2.7	2.7	2.6	2.5	2.5
NH ₃	2.0	2.0	2.0	2.0	2.1	2.0	2.0
Arg	7.9	7.6	7.3	6.4	7.9	6.8	6.2
Asp	12.1	11.9	11.9	11.7	11.9	11.3	11.4
Thr	4.2	4.1	4.2	4.1	4.1	3.9	4.0
Ser	5.6	5.5	5.6	5.5	5.6	5.4	5.4
Glu	18.9	18.5	18.7	18.7	18.8	17.8	18.2
Pro	5.3	5.2	5.2	5.2	5.2	5.1	5.2
Cys	1.7	1.8	1.5	1.2	1.7	1.4	1.3
Gly	4.2	4.1	4.2	4.2	4.4	4.2	4.3
Ala	4.3	4.2	4.3	4.3	4.3	4.2	4.1
Val	4.9	4.8	4.9	4.9	5.0	4.7	4.8
Met	1.2	1.3	1.2	1.3	1.3	1.3	1.4
Ile	4.5	4.5	4.5	4.6	4.6	4.3	4.0
Leu	8.1	7.9	8.0	8.0	8.1	7.7	8.2
Tyr	3.8	3.7	3.7	3.7	3.8	3.6	3.8
Phe	5.1	5.1	5.1	5.0	5.1	4.8	4.9
Trp	1.2	1.2	1.2	1.2	1.1	1.1	1.1

fused to eat their diet, and this part of the experiment was terminated after 3 days. A modified diet was designed specifically for this source of protein. The major change was that the ratio between unheated desolventized flakes and cod fillet was changed to 1:1 on a protein basis. After this change, the appetite improved, but still the mink wasted an average of 45 % of the diet. Mink fed the heated desolventized flakes or solvent extracted soybean meal took the entire ration with negligible refusals. The concistency of feces was satisfactory with all diets.

The true digestibility of nitrogen and amino acids in the mink study is shown in Table 3. The results show that the amino acid digestibility of the unheated desolventized flakes was very poor. However, there were considerable differences between individual amino acids; the arginine digestibility being relatively high while cystine and tryptophan were most poorly digested. Heating of desolventized flakes to 110 °C for 10 min. caused great increase in the digestibility of amino acids. For most amino acids the digestibilities exceeded significantly those of the commercial solvent extracted soybean meal. The amino acid digestibilities of desolventized flakes heated to 110 °C ranged from 77 (cystine) to 95 % (arginine), the

corresponding values for commercial solvent extracted soybean meal being 66 and 93 %. Heating to 135 °C caused a dramatic decline in the digestibility of all amino acids. The most affected amino acid was cystine, followed by aspartic acid, glycine, lysine and threonine. Arginine was the least affected amino acid. Thus the digestibility data revealed a selective effect of heat treatment on amino acid digestibility.

Table 3. Effect of heat treatment (temp./time) of soybean desolventized flakes and solvent extracted soybean meal on true nitrogen and amino acid digestibility in mink (%)

	Soybean desolventized flakes (°C/min.)				Solvent extracted soybean meal (°C/min.)			LSD
	-	110/10	135/10	135/30	-	135/10	135/30	
Lys	60	91	82	69	88	75	61	2
His	67	94	88	79	92	81	73	3
Arg	76	95	93	88	93	90	85	2
Asp	52	82	64	49	83	55	41	3
Thr	37	82	74	63	76	70	62	3
Ser	49	89	83	73	85	77	70	2
Glu	70	91	85	75	88	79	71	2
Pro	57	89	83	72	85	77	69	3
Cys	27	77	61	23	66	48	19	5
Gly	48	84	74	60	79	66	58	3
Ala	54	89	82	72	84	77	69	4
Val	50	90	84	75	86	78	72	2
Met	53	93	85	73	92	79	72	2
Ile	54	91	85	75	88	79	69	2
Leu	55	90	86	77	86	81	76	3
Tyr	61	93	88	80	87	83	77	4
Phe	47	84	79	72	77	76	70	3
Trp	20	89	83	72	86	76	66	4
N	51	89	81	70	84	75	66	2

The amino acid digestibilities obtained with chicks were mostly in good agreement with corresponding values for mink (Table 4). Cystine, threonine and phenylalanine appeared in general to be more efficiently digested by chicks than by mink. The effects of heat treatment were similar in both species. The low values for glycine are an artefact caused by hydrolysis of uric acid to glycine during the preparation of chick excreta for analysis.

The measurements of metabolizable energy with chicks revealed very low energy values for the unheated desolventized flakes and great improvement with heat treatment (Table 4). Energy utilization appeared to be less susceptible to overheating than amino acid digestibility. Auto-

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Table 4. Effect of heat treatment (temp./time) of soybean desolventized flakes and solvent extracted soybean meal on true amino acid digestibility (%) and metabolizable energy (ME) in chicks

	Soybean desolventized flakes (°C/min.)				Solvent extracted soybean meal (°C/min.)			LSD
	-	110/10	135/10	135/30	-	135/10	135/30	
Lys	64	91	82	62	93	76	60	5
His	62	93	58	74	95	80	73	5
Arg	63	96	93	87	96	92	86	4
Asp	55	89	73	50	92	66	50	6
Thr	55	88	81	71	91	76	67	5
Ser	50	92	84	73	93	80	70	6
Glu	62	92	86	75	94	83	75	5
Pro	53	90	83	69	94	81	68	7
Cys	46	85	70	44	89	62	46	9
Gly	-6	24	28	14	15	7	-1	13
Ala	47	87	79	67	89	75	65	5
Val	49	91	83	73	93	80	71	6
Met	40	92	83	70	95	78	73	8
Ile	46	92	84	72	93	81	69	6
Leu	48	91	85	75	93	82	75	6
Tyr	52	95	88	83	94	85	80	5
Phe	51	93	87	78	92	84	77	5
ME, MJ/kg DM	7.0	10.6	10.7	10.0	10.5	10.5	9.6	0.44

claving at 135 °C for 10 min. caused no reduction in content of metabolizable energy, in spite of the reduction in the digestibility of amino acids. However, heating at 135 °C for 30 min. caused significantly lower metabolizable energy values.

The results of the growth experiment with chicks are shown in Fig. 1. The unheated desolventized flakes failed to support satisfactory growth in chicks. Heat treatment at 135 °C resulted in inferior growth, feed efficiency and nitrogen utilization compared with the mild heating at 110 °C. Thus, the growth experiment verified the results of the amino acid digestibility measurements.

The effects of heat treatments on excretion of active trypsin in the two animal species are shown in Table 5. In mink, desolventized flakes increased the level of active trypsin in feces above the levels found with heated products. In chicks fed the desolventized flakes, no active trypsin was found in excreta. However, active inhibitors with ability to inhibit large amounts of chicken trypsin, were excreted. In both species the excretion of active trypsin increased with increased heating. The enzyme activity found in chick excreta was only about 1/20 of the activity in mink feces.

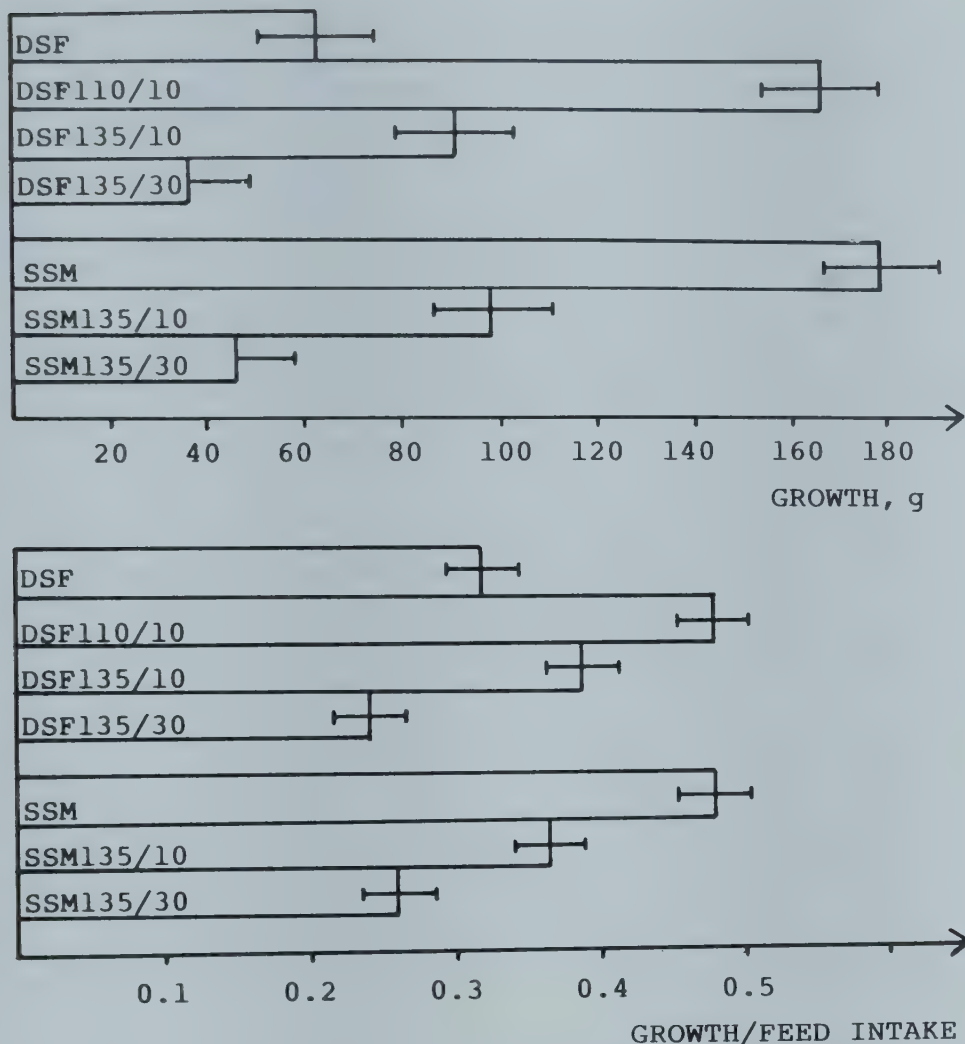


Figure 1. Effects of heat treatment of soybean desolventized flakes (DSF) and solvent extracted soybean meal (SSM) on growth and feed efficiency in chicks. The results represent the mean \pm SEM.

DISCUSSION

The limited use of soybean meal in mink diets is reflected by earlier research data, showing depressed growth and feed efficiency when appreciable quantities are included in the diet (6, 7, 8, 9, 10, 11). The basic reasons for the detrimental effects of soybean meal in mink diets remain obscure. One possible explanation is that mink has a unique sensitivity to small amounts of remaining proteinase inhibitors in commercial soybean meals. Thus it has been shown that additional heating, above that used in conventional soybean processing, may improve the nutritional value of soybean meal in fish diets (12).

Table 5. Effect of heat treatment (temp./time) of soybean desolventized flakes and solvent extracted soybean meal on trypsin activity* of feces from chicks and mink, OD₄₁₀/mg soybean meal intake

	Soybean desolventized flakes (°C/min.)				Solvent extracted soybean meal (°C/min.)		
	-	110/10	135/10	135/30	-	135/10	135/30
Chicks	-0.56 ^a	0.04 ^b	0.07 ^c	0.07 ^c	0.02 ^b	0.07 ^c	0.09 ^c
Mink	2.03 ^a	1.01 ^b	1.36 ^c	1.73 ^d	0.98 ^b	1.30 ^c	1.51 ^{cd}

* Results with different letters are significantly different ($p < 0.05$).

Amino acid digestibility is an important aspect to any protein source in diets for monogastric animals. Previous studies have revealed certain differences between mink and chicks with regard to ability of digesting the amino acids in different protein sources, the mink being more affected in case of lowered digestibility (2).

As early as in 1917, it was shown that soybeans should be heat treated in order to support normal growth (13). Raw soybeans contain proteinase inhibitors capable of reducing the digestibility of protein. On the other hand, excessive heating may cause loss of amino acids and lowered amino acid digestibility. Therefore it is important to aim at a balanced heat treatment, taking into account the necessary destruction of proteinase inhibitors as well as the possible adverse effects of overheating.

The results of the present study clearly demonstrate that raw soybean products, represented by unheated desolventized flakes with high levels of trypsin inhibitors, cause low amino acid digestibility in mink and chicks, and poor growth and feed efficiency in chicks. The low digestibility of raw soybean protein is caused by trypsin inhibitors and possibly by the resistance of undenatured soybean protein to enzymatic attack (14). Excessive secretory activity of pancreas would also contribute to low digestibility.

Mink fed unheated soybean flakes clearly had an excessive trypsin secretion. Preliminary analysis of total proteolytic activity in mink feces using casein as substrate, revealed activities 5 times the activity of feces from mink fed desolventized flakes heated at 110 °C. The other proteolytic enzymes were possibly excreted in even greater excess. The total amount of active enzymes present in feces cannot be estimated from the present results, since an unknown amount would be found to inhibitors and thereby escape analysis.

Accordingly, it is not possible to assess whether the total trypsin content in feces of chicks fed desolventized flakes is higher or lower than in the chicks fed flakes heated to 110 °C. It appears as if the chick pancreas, in contrast to the mink pancreas, was unable to compensate for the presence of large amounts of inhibitors in the diet. From the difference in fecal trypsin activity between the two species it might be speculated that the mink secretes more proteolytic enzymes in response to a diet than chicks.

Our findings support the work of Harada and coworkers (17), which compared the pattern of pancreatic enzyme secretion in herbivorous, omnivorous and carnivorous animals, including chicken and mink. Secretory responses, induced by vagal stimulation, cholecystokinin or intraduodenal infusion of a trypsin inhibitor, indicated differences between the groups of animals. Mink secreted proteinase-rich juice which was hormonally controlled whereas the chickens secreted amylase- and chymotrypsin-rich juice, which was less controlled by the vagus and hormones.

Soybean proteinase inhibitors and pancreatic proteolytic enzymes contain high levels of cystine and tryptophan, up to 18 and 2 %, respectively, in the inhibitors and up to 10 and 8 % in the proteinases. Excessive excretion of inhibitors and enzymes might, therefore, affect the digestibility of cystine and tryptophan more severely than that of other amino acids. The very poor tryptophan digestibility found with mink fed unheated desolventized flakes might have been due to a large excretion of proteinases. The low cystine digestibilities found with both species was to some extent due to the excretion of undigested inhibitors and enzymes.

The effects of heat treatment on amino acid digestibility revealed an overall similarity between mink and chicks. Both species responded with increased digestibility when desolventized soybean flakes were subjected to moist heat at 110 °C. Likewise, the performance of the chicks clearly showed the expected benefits of this heat treatment. Although there appeared to be no amino acid destruction by heating at 110 °C, the digestibility data indicated that some heat-sensitive amino acids would have been more available with less heating. This would also apply to the investigated sample of commercial solvent extracted soybean meal. However, whether or not this could be accomplished without an undesirable reduction in the inactivation of the trypsin inhibitors remains to be elucidated.

Moist heat treatment at 135 °C caused some browning, probably due to Maillard reaction products. This caused small losses of lysine, arginine and cystine, and a slight reduction in metabolizable energy. However, the deleterious effects of heating to 135 °C were much more pronounced

in terms of amino acid digestibility in mink and chicks, and growth retardation and poor feed efficiency in chicks. It is interesting to note that amino acid digestibility seems to be a more precise indicator of nutritive value than metabolizable energy and the chemically determined amino acid composition.

The present study accentuate the importance of proper heat treatment when soybean meal is used in diets for monogastric animals. In particular the effects on the digestibility of critical amino acids as cystine and lysine are of great importance. The results indicate species differences in physiological response to proteinase inhibitors. However, the study failed to confirm the hypothesis that the use of commercial soybean meals in mink diets may be limited by insufficient heating and remaining trypsin inhibitors.

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REFERENCES

1. Skrede, A. Utilization of fish and animal byproducts in mink nutrition. IV. Fecal excretion and digestibility of nitrogen and amino acids by mink fed cod (Gadus morrhua) fillet or meat-and-bone meal. Acta Agric. Scand. 29, 241 (1979).
2. Skrede, A., Krogdahl, A. and Austreng, E. Digestibility of amino acids in raw fish flesh and meat-and-bone meal for the chicken, fox, mink and rainbow trout. Z. Tierphysiol., Tierernährg. u. Futtermittelkde. 43, 92 (1980).
3. Muztar, A.J. and Slinger, S.J. A study of the metabolic fecal and endogenous amino acid excretion in fasted and mature cockerels with time. Nutr. Rep. Int. 23, 465 (1981).
4. Krogdahl, A. and Holm, H. Activation and pattern of proteolytic enzymes in pancreatic tissue from rat, pig, cow, chicken, mink and fox. Comp. Biochem. Physiol. 72A, 575 (1982).
5. Hammerstrand, G.P., Black, L.T. and Glover, J.D. Trypsin inhibitors in soy products: Modification of the standard analytical procedure. Cereal Chem. 58, 42 (1981).

6. Seier, L.C., Kirk, J.R., Devlin, T.J. and Parker, R.J. Evaluation of two dry protein sources in rations for growing-furring mink. Can. J. Anim. Sci. 50, 311 (1970).
7. Belzile, R.J. and Poliquin, L.S. Effects of feeding soya flour on the performance of growing-furring mink. Can. J. Anim. Sci. 54, 385 (1974).
8. Rimeslåttén, H. Soyamjøl som fôr til pelsdyr. Norsk Pelsdyrblad 48, 219 (1974).
9. Aldén, E. and Johansson, A.-H. Soyamjöl som foder till mink. Våra Pälsdjur 46, 75 (1975).
10. Skrede, A. Soybean meal versus fish meal as protein source in mink diets. Acta Agric. Scand. 27, 145 (1977).
11. Narasimhalu, P., Belzile, R.J. and Lepage, M. Effects of feeding raw meat or soybean meal on blood composition in mink (Mustela vison). Can. J. Anim. Sci. 58, 191 (1978).
12. Lovell, T. Using heat-treated full-fat soybean meal in fish feeds. Aquaculture Magazine 6 (3), 39 (1980).
13. Osborne, T.B. and Mendel, L.B. The use of soybean as food. J. Biol. Chem. 32, 369 (1917).
14. Kakade, M.L., Hoffa, D.E. and Liener, E.I. Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. J. Nutr. 103, 1772 (1973).
15. Blow, D.M. Stereochemistry of substrate binding and hydrolysis in the trypsin family of enzymes. In Proteinase Inhibitors. Proc. 2nd Int. Res. Conf., (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), Springer-Verlag, Berlin, p. 473 (1974).
16. Ikenaka, T., Odani, S. and Koide, T. Chemical structure and inhibitory activities of soybean proteinase inhibitors. Ibid., p. 325.
17. Harada, E., Nakagawa, K. and Kato, S. Characteristic secretory response of the exocrine pancreas in various mammalian and avian species. Comp. Biochem. Physiol. 73A, 447 (1982).

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DEVELOPMENT OF AN ARTIFICIAL CAECUM AND QUALITY OF THE OBTAINED PRODUCT

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SUMMARY

Considering the importance of the role played by the caecum in the rabbit, an artificial caecum was developed to allow the culture of caecal bacteria and to study the metabolic phenomena occurring. A yeast fermenter was adapted for the anaerobic growth of caecal bacteria in a semi-liquid medium composed of bran soaked in a nutrient liquor mainly contributing urea and minerals. The product obtained presented characteristics similar to those of the caecal medium (70 - 80 % water, 28 % TNM and 10^{10} microorganisms/g wt.). The release of acetic acid and the amino acid composition analysis indicate that the processes occurring in our artificial caecum were similar to those which take place in vivo. This model should not only allow us to cultivate caecal bacteria but also to determine the factors acting on their metabolism.

INTRODUCTION

Laboratory studies carried out into the physiology of rabbit digestion and nutrition have indicated the importance of the role played by the caecum (18 - 3 - 30) and experiments involving ligation of the pancreatic canal (6) and intracaecal urea perfusions (27) have pointed out the nutritional interest of the caecal flora in this animal. The use of germ-free animals confirmed this observation (32 - 33). Coprophagy and suppression of coprophagy show the microorganisms of the caecum represent 60 - 80 % of the soft pellet nitrogen content (13 - 18) and constitute a nonnegligible protein source for the rabbit (2 - 9) owing to the high protein content of the bacteria (15 - 33) this represents up to 20 % of total nitrogen intake of the rabbit (24). The need for quantities of caecal bacteria sufficient to allow their actual food value to be determined (preparation of diets differing in their total nitrogen-containing material (TNM = $N \% \times 6.25$) content and in the level of incorporation) led us to develop the artificial caecum.

This technique also presents the advantage of allowing the study of metabolic processes (bacterial proteosynthesis, cellulolytic activity, production of volatile fatty acids) and of the factors acting in the caecum (concentration of amino nitrogen, cellulose content and the ratio T.N.M./energy).

In this paper we shall present the development of the artificial caecum, the tests carried out and the composition of the products obtained.

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MATERIALS AND METHODS

Description and set-up of the apparatus

The artificial caecum was composed of a fermenter for semi-solid media used by the SETRIC Company to produce yeasts (28). It was made up of a vertical column of 13 l working capacity with a double boro-silicate glass wall allowing a thermostated liquid ($37^{\circ} - 38^{\circ}$) to circulate around the chamber. The two ends of the column were sealed with a stainless steel plate. The upper plate allowed access of the various probes (pH meter, circulation of gases, addition and removal of liquid). The lower plate was fitted with a sampling trap to enable inoculation of the bran and it also held a stirring rod with direct drive (Fig.1). An expansion vessel linked to the top and bottom of the chamber allowed circulation of the nutritional medium.

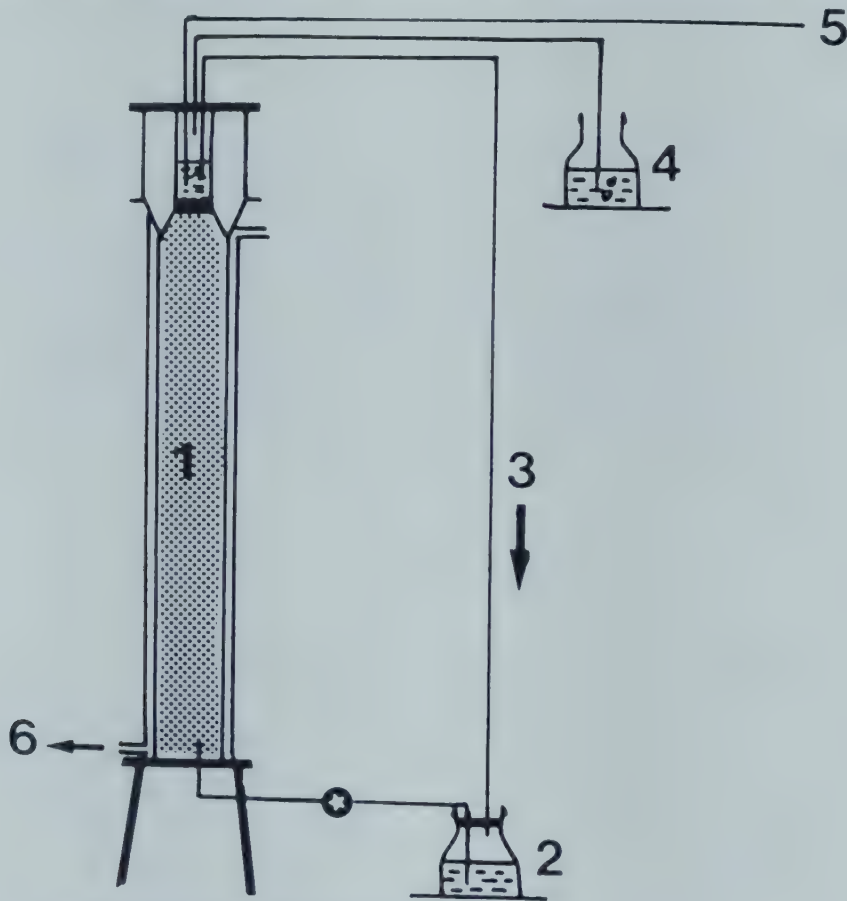


Figure 1. Set-up of the "artificial caecum".

- 1 - Column containing the bran soaked in nutrient solution
- 2 - Nutrient solution reservoir
- 3 - Direction of flow of the solution
- ⊛ - Nutrient solution pump
- 4 - Flask receiving the released gases containing a bacteriocidal solution
- 5 - pH meter
- 6 - water jacket outlet.

The fully fitted out fermenter is easily dismantled for sterilization in an autoclave but the circuits and culture medium were sterilized in situ with steam.

Once set up, the fermenter was air-tight and allowed culture of caecal bacteria in a semi-solid medium composed of bran soaked in a nutrient solution in strictly anaerobic conditions obtained, before fermentation, by flushing with nitrogen to remove the air from the apparatus. The bran and nutritive medium were autoclaved before use.

Culture medium

Three kg of sterilized bran were placed in the vertical column of the fermenter and covered with mesh weighed down with glass balls to retain it during the circulation of the nutritive solution.

The bran was then soaked in 9 l of sterile nutritive solution kept in continuous circulation by a system using a sufficiently powerful pump. The nutritional medium (Table Ia) was prepared in such a way that its composition was close to that of the caecal liquor at least for the nitrogen, free sugar and mineral content (5).

Table I

a) Composition (g/l) of the nutrient solution

Mineral mix	5
Sucrose	5
$(\text{NH}_4)_2 \text{PO}_4$	2
$(\text{NH}_4)_2 \text{SO}_4$	2
Peptones	1
Urea	10

b) Composition (g/kg) of the mineral mix (UAR n° 205)

Ca H PO_4	430
KCl	100
NaCl	100
Mg Cl_2	50
Mg SO_4	50
$\text{Fe}_2 \text{O}_3$	30
$\text{Fe}_2 \text{SO}_4 (7 \text{ H}_2\text{O})$	50
Oligoelements	10

The soluble and fermentable nitrogen (7 g/l) - 70 % of which was in the form of urea - represents an equivalent of 3.7 g soluble T.N.M. per 100 ml of liquor or 11 % dissolved dry matter. These values are close to those given by Marty who found, at the entry to the caecum, 1840 μ mol total amino acids per 100 g liquid ; this represents up to 10 % T.N.M. in dry wt. This concentration of soluble nitrogen corresponds to the optimum level of microorganism growth in the rumen (22).

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The energy necessary to start off the fermentation was supplied by sucrose - it represented 1.5 % of the dry wt : this value is similar to the 1 - 2 g/100 g dry wt of free sugars found in the caecum output supernatant (5).

The mineral mix (Table Ib) was that used by the manufacturer of laboratory diets (U.A.R.) supplemented with phosphate and sulphate in the form of their ammonium salts to encourage bacterial growth (4). Phosphate is required for the synthesis of nucleic acids which contain 10 - 20 % of the bacterial nitrogen, and sulphur for the synthesis of methionine and cystine present in the microbial proteins.

The peptones were those usually used in microbiological cultures.

The mixture was therefore composed of bran soaked in nutritive medium with 70 - 80 % water and a T.N.M. content, 45 % of which is soluble, representing 25 % of the dry matter - it was similar in composition to the caecal contents. It should allow an acceptable level of bacterial growth.

Use of the artificial caecum

The bran and the nutrient medium were placed in the column of the fermenter and the circulation circuit after sterilization.

The whole system was flushed with nitrogen to displace the oxygen and so create the anaerobic conditions necessary for the development of the caecal flora (12).

The column was inoculated with the caecal contents of a healthy adult rabbit fed on commercial diet. The fresh caecal matter was introduced at the base of the column via the sampling trap.

The fermentation temperature was maintained at 37° - 38° with the thermostated water-jacket. The nutrient liquid was circulated with an ASTI pump : this spreads the inoculating caecal contents throughout the column - mixing was achieved with the stirring rod.

During the whole period of fermentation (4 - 5 days) the pH fell due to release of volatile fatty acids arising from the activity of the microbial population (3 - 30 - 23).

It was regularly brought to between 6 and 7 by addition of sodium hydroxide and the gas released was bubbled through a N sodium hydroxide solution which is bacteriocidal and which traps the volatile fatty acids released in the form of their sodium salts for later assay.

The product obtained at the end of the fermentation was composed of fermented bran soaked in nutrient solution which was to be assayed and 1 - 2 liters nutrient solution in the circulation system (about 1/10 th of the initial volume) which must be taken into consideration in the yield calculations.

Product analysis techniques

1.- The total flora density at the end of fermentation was determined by direct counting after dilution and staining with nigrosine. The number of microorganisms (both viable and non-viable) was determined under the microscope (x 500) after spreading the diluted suspension on the central zone of a PRECIS counting slide (5).

2.- The anaerobic bacteria were counted by inoculation of deep tubes (8 mm x 400 mm) containing gelose, meat-yeast medium enriched with glucose (25) under carbon dioxide atmosphere.

3.- The density of the cellulytic flora was evaluated using Hungate's method in roll tubes, under carbon dioxide atmosphere with a strip of filter paper as the only energy source (14).

4.- The volatile fatty acids released were assayed in the liquid medium by gas chromatography (Varian Aerograph 2100 and PEG.4000 column).

5.- The bran and products at the end of fermentation were soaked in distilled water (50 g dry wt in 0.5 l) and magnetically stirred for 1 h allowing the bacteria to separate from the plant debris which was removed, at least the larger particles, by filtration through cloth. The filtrate still contained microscopic plant residues which were eliminated on slow centrifugation (500 g). The microorganisms were extracted from the supernatant by fast centrifugation (28000 g). For the bran (fermented or not), the various supernatants, the plant residues and bacterial sludge, measurements were made of the dry wt. (after 24 h in an oven at 110°), the level of nitrogen (by Kjeldahl's method) and the various amino acids (with a Beckman automatic analyser after hydrolysis in 6 N HCl).

RESULTS

1.- Throughout the whole fermentation period large amounts of gas were released and the pH fell owing to the presence of valatile fatty acids. The circulating liquid was found to countain 10-15 mM acetic acid and traces of propionic and butyric acid (<2mM).

2.- The product obtained at the end of fermentation presented the following characteristics : total number of bacteria : 10^{10} /g fresh mix water : 70 - 80 %.

total Nitrogen-containing Material (T.N.M.) : 25 - 32 % dry wt.

number of living anaerobic bacteria : 10^7 - 10^9 /g fresh mix.

number of living cellulolytic bacteria : 10^3 - 10^4 /g fresh mix.

The mix was composed of the natural balance between residual bran and the bacteria growth.

3.- Microscopic observation of the pellet from the second centrifugation (28000 g) showed that it was a real bacterial sludge with very little plant debris. The different species of bacteria were not identified systematically but the sludge was seen to be mainly composed of Bacteroides and Coliforms.

4.- The level of Total Nitrogen-containing Material (T.N.M.), protein and the amino acid distribution were analysed in the bran, the fermented bran, the bran supernatant, the fermented bran supernatant, the plant debris and the bacterial sludge (Table II).

The non fermented bran showed a T.N.M. level of 12.8 %. After soaking of non-fermented bran and centrifugation at 500 g, a pellet was obtained composed of large particles of plant debris and a supernatant containing, per 100 ml, 17 mg of T.N.M. for a dry wt. of 160 mg (11 %) or, in another way 7 mg of T.N.M. per g dry wt. soaked non-fermented bran.

The mean with S.E. for T.N.M. of fermented bran (% of dry wt) reached $28 \pm (1.1)$. After soaking and centrifugation at 500 g, the plant debris pellet showed a T.N.M. level of about 13% and 100 ml supernatant presented 350 mg of T.N.M. for the 620 mg dry matter i.e. 56 %.

Table 2. Proportions of the various amino acids (in % and in rug/g dry wt.), total of the amino acids analysed, Level of ammonia (in mg/g dry wt.), ammonia T.N.M. equivalent (N x 6.25) and T.N.M. content (Parnass assay) of Non-Fermented Bran, Fermented Bran, their corresponding 500 g supernatants (obtained on centrifugation at 500 g of a mixture of 50 g bran in 500 ml water ; in this case, the results are given in rug amino acid per g dry weight of the corresponding bran put to soak), the dry matter of the fermented bran 500 g supernatant, the fermented bran 500 g pellet (bran debris) and the fermented bran 28 000 g pellet (bacterial sludge obtained after centrifugation of the 500 g supernatant at 28 000 g).

	Non Fermented		Fermented		500 g		500 g		DM of		500 g		28 000 g	
	Bran		Bran		Supernatant		Supernatant		Supernatant		pellet		pellet	
	(NFB)	(FB)	(NFB)	(FB)	(NFB)	(FB)	(NFB)	(FB)	(FB)	(FB)	(bran debris)	(bacterial sludge)	(bacterial sludge)	(bacterial sludge)
	%	mg/g DM	%	mg/g DM	%	mg/g DM	%	mg/g DM	%	mg/g DM	%	mg/g DM	%	mg/g DM
Asp	9	10	9	19	9	0,3	8	6	8	27	9	12	6	17
Thr	4	4	5	8	4	0,2	3	2	3	10	5	5	7	12
Ser	6	5	6	9	6	0,2	4	2,5	4	11	6	6	7	11
Glut	15	19	17	37	19	1	20	16	20	72	15	20	17	27
Pro	7	7	6	9	8	0,3	9	10	9	45	7	7	7	8
Gly	10	6	9	11	11	0,2	10	4	9	18	9	6	9	11
Ala	9	7	10	12	9	0,3	10	5	10	23	9	7	11	19
Val	7	6	7	12	6	0,2	5	3,5	5	16	7	7	6	8
Cys	1	1	1	3	1	0,04	1	1	1	4	1	1	1	3
Met	1	2	1	2	1	0,02	1	0,5	1	3	1	2	1	2
Ileu	4	4	4	8	3	0,2	3	2,5	3	11	4	5	5	11
Leu	8	9	8	14	6	0,2	6	4	6	18	8	10	8	18
Tyr	2	3	2	5	1	0,1	1	1	1	5	2	3	2	7
Ala	3	5	3	9	3	0,2	3	3	3	13	3	6	3	8
NH ₂ But	2	2	2	2	4	0,1	6	5	6	22	2	2	4	14
Orn	1	1	1	2	1	0,06	3	2	3	9	1	1	2	4
Lys	4	5	5	10	4	0,2	4	3	4	14	4	6	6	16
His	2	3	2	5	1	0,07	1	1,5	1	6	2	3	1	3
Arg	5	8	4	10	3	0,1	3	3	3	13	5	9	3	10
Total	100	109	100	187	100	4,0	100	84,5	100	340	100	118	106	209
NH ₃		2		10		0,3		5		27		2		17
N(NH ₃) x6.25		12		62		1,8		31		140		12		88
T.N.M.	12,8	128	28,2	282	0,02	7	0,36	140	56	560	13	130	33	330

So, per g dry wt. fermented bran 250 mg dry extract containing 140 mg T.N.M. are solubilized. On average this means that about half the total T.N.M. and a quarter of the dry wt. of the fermented bran are found in the supernatant.

The product of the artificial caecum is therefore seen to be twice as rich in T.N.M. as non-fermented bran and the corresponding supernatant contained twenty times more T.N.M. for a dry wt. four times greater. When the first 500 g supernatant of the fermented bran was recentrifuged at 28000 g, the pellet was composed of a bacterial sludge containing small quantities of microscopic plant debris -its T.N.M. content was about 33 % dry wt.

5.- Amino acid distribution analysis showed that with non-fermented bran, in both the whole bran and the supernatant, the two amino acids found in the highest proportions were glutamic acid and glycine whereas for the fermented bran, the corresponding supernatant and the bacterial sludge the commonest were glutamic acid and alanine.

The least frequent amino acids were, before fermentation, ornithine and the thio amino acids and after fermentation the same with histidine. The ratio fermented to unfermented bran for each amino acid (Table II); shows that on fermentation there is enrichment of threonine, valine, isoleucine, ornithine and lysine and a drop in arginine, proline and γ -amino butyric acid.

In the bacterial pellet, the most frequent amino acids (%) were glutamic acid (17), alanine (11), glycine (9) and the least frequent cystine and methionine (1), histidine (1), ornithine (2) and tyrosine (2).

In each case, the sum of the amino acids is lower than the level of TNM. The difference can be explained by the losses due to the technique used for the assay. Acid hydrolysis completely destroys tryptophan and deaminates glutamine and asparagine causing release of large amounts of nitrogen in the form of ammonia.

6.- However, it should also be considered that, for the fermented bran, the volume of nutrient liquid remaining in the circuit represents a loss of 40.10^3 mg of T.N.M. and of 25.10^3 mg of amino acid i.e. when expressed per g dry wt. fermented bran : 16 mg T.N.M. and 8-12 mg amino acid. Finally, part of the nitrogen which can be evaluated at a T.N.M. equivalent of 25 mg/g dry wt. is in the form of bacterial nucleic acids (for the supernatant of the fermented bran and for the bacterial sludge the proportion is certainly much higher). When the total level of T.N.M. is calculated taking these remarks into consideration a figure very close to that obtained by direct assay is reached.

7.- At the end of the fermentation, the loss of bran mass was about 30 % for a microorganism production of 50 - 130 g.

DISCUSSION

The technique of fermentation in semi-solid medium, based on direct protein enrichment of solid agricultural industry by-products (bran, urea), does not require as much equipment as fermentation in liquid medium ; it avoids the problems of dilution and extraction of the biomass since, in this case it is the whole medium which is directly collected and used - it also allows the anaerobic conditions necessary to be obtained more easily.

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The mixture composed of bran soaked in nutrient medium is composed in such a way that, for many parameters, it is similar to the caecal contents - it allows acceptable bacterial growth in conditions close to those which exist in the live animal.

During the fermentation, the release of volatile fatty acids and especially acetic acid - which is a major end product of bacterial metabolism in the hindgut of rabbit (1 - 3 - 31) - indicates the strong development of flora and a certain analogy with what happens in the caecal medium (30).

The product obtained is a bacterial concentrate (10^{10} /g matter) with levels of water, nitrogen and microorganisms close to that found in caecotrophs (5 - 6).

The flora grown in this way can have a composition slightly different to that of the inoculation flora owing to the development of certain microbial species at the cost of others yet the preponderance of Bacterioides and Coliforms observed does resemble the situation described by Martin (17) and Gouet & Fonty (12) for caecal flora.

Amino acid analysis gives further data (Table II). The distribution of the amino acids in non-fermented bran is in agreement with the results published by the N.R.C. (21). It is the same for the supernatant and the debris pellet. With fermented bran however the proportions of aspartic acid and alanine were seen to be slightly higher : these two amino acids are fairly frequent in microorganisms and soft pellets (11 - 10 - 24 - 9). The same amino acids are found in large quantities in the supernatant of the fermented bran and also in the bacterial sludge, the most common essential amino acids being lysine and leucine.

Fermented bran is in fact a mixture of bacteria and bran where over half the T.N.M. (54 %) is of bacterial origin and the amino acid distribution of the ferment is the sum of the composition of the bran and of the microorganisms.

When soaked, fermented bran releases a large quantity of amino acids (twenty times more than non-fermented bran) into the aqueous phase. Their origin is mainly the bacteria found in the resulting suspension : proline is the second most abundant amino acid. Considering the losses due to the assay technique used and the nitrogen contained in the nucleic acids of the microorganisms, 85-90 % of the nitrogen in the supernatant is in the form of amines and all the non-protein nitrogen (urea and ammonium sulphate and phosphate) has been used in bacterial synthesis.

After the first centrifugation at 500 g the supernatant containing the bacteria is separated from the pellet in which a large amount of microorganisms remain attached to the bran debris. The bacteria are obtained from the supernatant by centrifugation at 28000 g but the resulting pellet still contains a certain quantity of plant residue. The high-speed supernatant contains the amino acids of bacterial metabolism released into the medium.

The similarity between our results and those obtained by Megard (19) Yoshida *et al.* (32), Djoukam (10) and Proto (24) on the amino acid composition of rabbit caecotrophs suggests that our experimental conditions are closed to those existing in the caecum and the comparison of our values with the proportions of free amino acids reported by Marty *et al.* (18) in plant residue, caecal liquor and the bacterial cells confirms the analogy between what happens *in vitro* and *in vivo*. The yield obtained is quite acceptable : experimental results show that, in the rumen, microbial synthesis is variable and fluctuates between 90-230 g/kg organic material digested (4).

An artificial caecum has been developed which allows the in vitro

cultivation caecal bacteria in a semi-liquid medium composed of bran soaked in a nutrient solution containing urea as the main nitrogen source. The present study does bear an analogy with the rumen simulation technique of Czerkowski and Breckenridge (8) and exposes the same phenomena as those described by Mehra (20) on incubation of microorganisms taken from ruminants.

The bacteria which obtain their energy from the polysaccharide structures of the bran use, for their protein synthesis, all the non-protein nitrogen of the nutrient medium as well as part of the plant proteins and the product obtained at the end of fermentation has a composition analogous to that of the caecal contents.

This model - which creates conditions very close to those which exist in the rabbit caecum - has allowed the preparation of a product which, owing to its resemblance to the soft pellets must constitute a foodstuff which is extremely advantageous for the rabbit. It can lead to a fuller study of the metabolic processes which occur in the caecum and especially can allow research into the factors favourizing, in this digestive compartment, the bacterial protein synthesis (amonia concentration, available energy and the energy/nitrogen ratio) and the use of cellulose (17 - 26). It could also be used in an attempt to modify the flora on culture of caecal bacteria (eg. development of cellulolytic flora).

REFERENCES

- 1 - Bailey, J. Mc Bee, R.M.. The magnitude of the rabbit caecal fermentation. *Proc. Montana Acad. Sci.* 24, 35-38, 1964.
- 2 - Battaglini, M.B. Importanza della coprofagia nel coniglio domestico in rapporto alla utilizzazione di alcuni principi nutritivi. *Riv. Zootech. Agric. Vet.*, 6, 21-37, 1968.
- 3 - Beauville, M., Raynaud, P., Vernay, M. Concentration des acides gras volatils plasmiques chez le lapin. *Ann. Rech. Vétér.*, 5, 407-411, 1974.
- 4 - Bondi, A. Metabolism of protein in ruminant animals : a review. *Nutr. Rep. Int.*, 23, 993-1004, 1981.
- 5 - Bonnaïous, R., Raynaud, P. Recherches sur le rôle du colon dans la dualité de l'excrétion fécale du lapin. *Arch. Sc. Physiol.*, 21, 261-270, 1967.
- 6 - Catala, J. Effet de la ligature du canal pancréatique chez le lapin nourri traditionnellement. *Ann. Biol. anim. Bioch. Biophys.*, 17, 573-578, 1977.
- 7 - Colin, M. Contribution à l'étude des besoins en acides aminés essentiels du lapin en croissance. Thèse de Docteur Ingénieur, Université des Sciences et Techniques du Languedoc, Montpellier, 1978.
- 8 - Czerkowski, J.W., Breckenridge, G. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.*, 38, 371-384, 1977.
- 9 - Demaux, G., Gallouin, F., Guemon, L., Papantonakis, C. Effets de la privation prolongée du comportement de caecotrophie chez le lapin. *Reprod. Nutr. Develop.*, 20, 1651-1659, 1980.
- 10 - Djoukam, J. Caecotrophie et nutrition azotée du lapin. Thèse de Doctorat de 3ème Cycle, Université Paris VI, 1973.
- 11 - Ferrando, R., Wolter, R., Vitat, J.C., Megard, J.P. Teneur en acides aminés des deux catégories de fèces du lapin : caecotrophes et fèces dures. *C. R. Acad. Sci., Paris*, 270, 2202-2204, 1970.

NUTRITION REPORTS INTERNATIONAL

- 12 - Gouet, P., Fonty, G. Changes in the digestive microflora of holoxenic rabbit from birth until adulthood. *Ann. Biol. anim. Bioch. Biophys.*, 19, 553-566, 1979.
- 13 - Griffiths, M., Davies, D. The role of the soft pellets in the production of lactic acid in the rabbit stomach. *J. Nutr.*, 80, 171-180, 1963.
- 14 - Hyngate, R.E. The rumen and its microbes. Academic Press, Inc. New-York and London, 1966.
- 15 - Kihlberg, G. The microbe as a source of food. *Ann. Rev. Microbiol.*, U.S.A., 26, 427-466, 1972.
- 16 - Lebas, F. Influence de la teneur en énergie de l'aliment sur les performances de croissance chez le lapin. *Ann. Zootech.*, 24, 281-288, 1975.
- 17 - Martin, C. Contribution à l'étude de la flore caecale du lapin. Essai d'implantation de lactobacilles. Thèse de Docteur Vétérinaire, Lyon, 1971.
- 18 - Marty, J., Raynaud, P., Carles, J. Les acides aminés et les acides carboxyliques dans le caecum du lapin. *Ann. Biol. anim. Bioch. Biophys.*, 13, 429-451, 1973.
- 19 - Megard, J.P. Aspect alimentaire de la caecotrophie chez le lapin. Thèse de Docteur Vétérinaire, Paris, 1970.
- 20 - Mehra, U.R. Incubation of hay with rumen microorganisms ; two distinct phases of digestion. *Proc. Nutr. Soc.*, 40, 1981.
- 21 - National Research Council. Nutrient Requirements of Rabbits. National Academy of Sciences, Washington, 1977.
- 22 - Okorie, A.U. Microbial protein synthesis in relation to rumen and duodenal ammonia concentrations. *Nutr. Rep. Int.*, 24, 1241-1249, 1981.
- 23 - Parker, D.S. The measurement of production rates of volatile fatty acids in the caecum of the consciencious rabbit. *Br. J. Nutr.*, 36, 61-70, 1976.
- 24 - Proto, V. Fisiologia della nutrizione del coniglio con particolare riguardo alla ciecotrofia. *Coniglicoltura*, 7, 15-33, 1976.
- 25 - Raibaud, P., Dickinson, A., Sacquet, E., Charlier, H., Mocquot, G. La microflore du tube digestif du rat. I - Technique d'étude et milieux de culture proposée. *Ann. Inst. Pasteur*, 110, 558-590, 1966.
- 26 - Rossilet, A. La cellulose dans l'alimentation du lapin : utilisation digestive et aspects nutritionnels. Thèse de Docteur Ingénieur, Université Pierre et Marie Curie, Paris VI, 1977.
- 27 - Salse, A., Crampes, F., Raynaud, P. Valeur nutritionnelle de l'urée donnée en perfusion intracaecale chez le lapin. *Ann. Biol. anim. Bioch. Biophys.*, 17, 559-565, 1977.
- 28 - Sétric Génie Industriel, B.P. 4050 - 31029 Toulouse Cédex
- 29 - Usine d'Alimentation Rationnelle, 7, rue du Maréchal Galliéni, Villemoisson-sur-Orge - 91360 Epinay-sur-Orge.
- 30 - Vernay, M., Raynaud, P. Répartition des acides gras volatils dans le tube digestif du lapin domestique. *Ann. Rech. Veter.*, 6, 357-377, 1975.
- 31 - Woodnut, G., Parker, D.S. Absorption of acetate and butyrate from the caecum of the rabbit. *Proc. Nutr. Soc.*, 40, 17 A, 1981.
- 32 - Yoshida, T., Kandatsu, M. Studies on caecum digestion. On the compo-

NUTRITION REPORTS INTERNATIONAL

sition of the digestive tracts contents in the rabbits during night time. Jap. J. Zootech. Sci., 39, 299-305, 1968.

- 33 - Yoshida, T., Pleasants, J.R., Reddy, B.S., Wostmann, B.S. Amino acid composition of caecal contents and feces in germ free and conventionnal rabbits. J. Nutr., U.S.A., 101, 1423-1429, 1971.

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BOOKS RECEIVED

LABORATORY MANUAL FOR DATA PROCESSING IN ASSESSMENTS OF DIETARY PROTEIN QUALITY BY AN UPDATED VERSION OF THE NITROGEN METABOLISM METHOD. J.J. Dreyer and W.H. van der Valt. 145 pages, \$50.00. National Food Research Institute (CSIR), P.O. Box 395, Pretoria 0001 South Africa.

